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## DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

### XIII. THE STABILITY OF HYDROGEN IN AMINO ACIDS\*

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If carbon-bound hydrogen of physiological substances is replaced by deuterium, the metabolism of such compounds, after their administration to an animal, can be studied by tracing the route taken by the deuterium. The investigation of the metabolism of fatty acids and sterols is facilitated by the great stability of their carbon-bound hydrogen, which as has been shown does not exchange with the hydrogen of water, even when the substances are heated in alkaline or acid solution. The experimental procedure developed for fatty acids and sterols, however, cannot be applied to the study of amino acid metabolism until the stability of their hydrogen has been investigated.

Amino acids contain at least two polar groups at neighboring carbon atoms, an amino and a carboxyl group. The hydrogen atoms in these are ionizable and exchange instantly when dissolved in water. The deuterium technique is not applicable to these groupings, as the isolation procedure removes all deuterium which might have been present in them. These and other polar groups (OH,—SH, etc.), however, may conceivably labilize adjacent carbon-bound hydrogen atoms. In some compounds the chemical reactivity of hydrogen atoms adjacent to polar groups has long been recognized by organic chemists, and advantage has been taken of this lability in many synthetic procedures. The reactivity, as well as the rate of exchange, of such hydrogen atoms

\* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

varies considerably from compound to compound. Thus, for example, the  $\alpha$ -hydrogen atoms of malonic acid exchange easily (1), and a slow exchange takes place with sorbic and crotonic acids (2), while those of the higher saturated fatty acids are very stable towards exchange. The lability of hydrogen adjacent to carbonyl groups (enolizable hydrogen) has frequently been discussed. Experience with exchange reactions is still too limited to permit one to predict, from a knowledge of the structure of a given compound, which carbon-bound hydrogen atoms are exchangeable, and how fast this reaction proceeds. All such exchange reactions of carbon-bound hydrogen are in general much slower than that of the ionizable hydrogen. For convenience, we shall call such hydrogen which exchanges slowly with the hydrogen of water "semilabile hydrogen."

We have investigated the behavior of the hydrogen of a number of amino acids in boiling aqueous acids, as in protein hydrolysis, which is the most drastic procedure to which amino acids are subjected during their isolation from biological material.

The stability of hydrogen in an organic compound can be investigated by two experimental procedures: A, by treating a deuterio compound with ordinary water, or B, by treating the ordinary (non-deuterium-containing) compound with heavy water. Experiments involving both procedures have been carried out on several amino acids.

*Procedure A*—*dl*-Leucine- $\beta,\gamma$ - $d_2$  (3), *dl*-valine- $\beta,\gamma$ - $d_2$  (3), homocystine- $\beta,\gamma$ - $d_2$  (4), methionine- $\beta,\gamma$ - $d_4$  (4), deuterio-*dl*-leucine, and deuterio-*dl*-alanine prepared with deuteriosulfuric acid (5) were investigated.<sup>1</sup> With the exception of the methionine, which lost a small fraction (about 6 per cent) of its original deuterium, these compounds proved stable on long boiling with 20 per cent HCl. The exception may be related to the decomposition which occurs when methionine is treated under the conditions which we have employed. The results definitely prove that the hydrogen atoms at the  $\beta$  and  $\gamma$  positions in leucine, valine, homocystine, and most probably methionine are stable towards exchange.

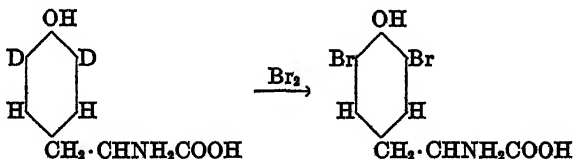
*Procedure B*—The following normal amino acids were refluxed

<sup>1</sup> The authors are deeply indebted to Professor Roger Adams for the samples of leucine and valine, and to Professor V. du Vigneaud for the homocystine and methionine.

with 20 per cent hydrochloric or sulfuric acid in dilute heavy water: glycine, proline, phenylalanine, tyrosine, cystine, glutamic acid, and lysine.

In agreement with the results of Günther and Bonhoeffer (6) and Krogh and Ussing (7), the hydrogen of the methylene group of glycine is semilabile. When treated under our conditions, glycine took up deuterium. By our procedure, no deuterium was introduced into lysine, and only traces into phenylalanine and proline. Appreciable amounts were found in tyrosine, cystine, and glutamic acid.

The reactivity of carbon-bound hydrogen in the last three amino acids was expected on the basis of their structure and chemical reactions. The ortho hydrogen atoms in phenols are highly reactive, and their exchangeability has recently been demonstrated by Best and Wilson (8). Tyrosine contains a phenolic group. That the deuterium from the heavy water entered into the ortho position could be proved by bromination; the resulting dibromotyrosine contained no deuterium.



The fact that glutamic acid in acid solution is in equilibrium with pyrrolidonecarboxylic acid may be responsible for the observed uptake of deuterium. Our findings on cystine confirm those of Stekol and Hamill (9). After the treatment with strong mineral acids, our sample, as well as that of these authors, was appreciably racemized. This is probably an effect of the same mechanism as that by which the deuterium was introduced. Krogh and Ussing (7) found racemization of proteins, when carried out in heavy water, to lead to an introduction of deuterium into its amino acids, and Erlenmeyer and coworkers (10) observed uptake of deuterium into *L*-menthyl phenylbromoacetate in the course of its racemization. Both authors suggest that the introduction occurred at the  $\alpha$ -carbon atom, the center of asymmetry.

Stekol and Hamill (9) have reported that the hydrochlorides of

lysine, as well as arginine and histidine, take up small amounts of deuterium from heavy water at 150°. As stated above, under our conditions (in 20 per cent HCl at 108.6°), lysine showed no such exchange. The temperature employed by Stekol and Hamill is much above that ordinarily employed in work with proteins and amino acids, and their results, while interesting, do not apply to biochemical work. It is not unlikely that at sufficiently high temperature all organic compounds exchange their carbon-bound hydrogen atoms.

The introduction of deuterium from heavy water into the amino acid molecule is *reversible*. The deuterium can be removed by treatment with acid in ordinary water. Stekol and Hamill have shown this for their cystine preparation. We confirm their findings and show the same for glutamic acid.

The results indicate that, with the exception of glycine, which contains only one kind of carbon-bound hydrogen, amino acids contain stably bound hydrogen which does not exchange with the hydrogen of water under conditions of acid hydrolysis. This is of greatest importance for the application of the deuterium technique for the study of amino acid metabolism. The presence of semilabile deuterium should not interfere with the procedure if suitable methods are employed.

#### EXPERIMENTAL

##### *Preparation of Deuteroamino Acids with D<sub>2</sub>SO<sub>4</sub><sup>2</sup>*

*Deutero-dl-Alanine*—1.5 gm. of *dl*-alanine were dissolved in 3 cc. of 91.6 per cent D<sub>2</sub>SO<sub>4</sub> containing 25 atom per cent of deuterium. The mixture was heated in a sealed tube at 100° for 48 hours. The slightly yellow solution was poured into 400 cc. of water and neutralized with barium hydroxide. The filtrate was brought to dryness, and the residue was redissolved in 50 cc. of water and precipitated with alcohol. The precipitation was repeated. 1.107 gm. were obtained containing 15.65 per cent N and  $1.30 \pm 0.02$  atom per cent deuterium.

*Deutero-dl-Leucine*—1.5 gm. of *l*(-)-leucine ( $[\alpha]_D$  in 20 per cent HCl = +18.8°) were treated as above. After removal of the sul-

<sup>2</sup> A preliminary note dealing with this reaction has already been published (5).

furic acid with  $\text{Ba}(\text{OH})_2$ , the leucine was twice recrystallized from hot water. 1.04 gm. were obtained.  $[\alpha]_D$  in 20 per cent  $\text{HCl}$  =  $0.0^\circ$ . The substance contained 10.53 per cent N and  $0.86 \pm 0.02$  atom per cent deuterium.

### *Stability of Hydrogen in Amino Acids*

#### *Procedure A. Treatment of Deuteroamino Acids with $\text{H}_2\text{O}$*

*Deutero-dl-Alanine*—698 mg. of the above deutero-*dl*-alanine were boiled for 48 hours with 45 cc. of 20 per cent ordinary sulfuric acid. The acid was removed with  $\text{Ba}(\text{OH})_2$  and the alanine was isolated from the filtrate as mentioned above. 533 mg. were obtained. The deuterium content was unchanged ( $1.30 \pm 0.02$  atom per cent).

*Deutero-dl-Leucine*—500 mg. of the above deutero-*dl*-leucine were treated with 20 per cent ordinary sulfuric acid in the same way as alanine. The deuterium content was unchanged ( $0.86 \pm 0.02$  atom per cent).

*dl-Leucine- $\beta, \gamma$ - $d_2$* —0.286 gm. of the substance was dissolved in 15 cc. of  $\text{H}_2\text{O}$  and refluxed for 4 days. 15 cc. of concentrated  $\text{HCl}$  were added and boiling was continued for 4 days more. The solution was evaporated to dryness, the residue redissolved in water, the water evaporated, and the procedure repeated. The residue was taken up in hot alcohol and the solution was filtered. On neutralization with alcoholic aniline, the leucine crystallized. 0.234 gm. of substance was recovered. The deuterium content before treatment was  $13.6 \pm 0.3$  and after treatment  $13.7 \pm 0.3$  atom per cent.

*dl-Valine- $\beta, \gamma$ - $d_2$* —0.429 gm. of substance was treated exactly as above. 0.308 gm. was recovered. The deuterium content before treatment was  $14.4 \pm 0.3$  and after treatment  $14.5 \pm 0.3$  atom per cent.

*dl-Homocystine- $\beta, \gamma$ - $d_2$* —A mixture of 47.5 mg. of the deutero compound together with 231.8 mg. of ordinary *dl*-homocystine was boiled for 48 hours with 20 per cent hydrochloric acid. No decomposition was observed. The solution was evaporated under reduced pressure, the residue redissolved in water, and the solution again evaporated. The residue was dissolved in 95 per cent alcohol and the homocystine precipitated by neutralization with

aniline. 270 mg. were recovered. The deuterium content of the mixture before treatment was  $4.11 \pm 0.07$  and after treatment  $4.17 \pm 0.07$  atom per cent.

*dl-Methionine- $\beta,\gamma$ - $d_2$* —46.7 mg. of the deuterio compound together with 201.7 mg. of ordinary *dl*-methionine were refluxed for 24 hours with 20 per cent HCl. A small amount of white material deposited in the inside of the condenser. The solution had a slight odor. The isolation was carried out as with homocystine and 230 mg. of substance were recovered. The deuterium content of the mixture before treatment was  $3.33 \pm 0.03$  and after treatment  $3.14 \pm 0.05$  atom per cent.

*Procedure B. Treatment of Ordinary Amino Acids with Heavy Water*

*Glycine*—3 gm. of glycine in a mixture of 5 cc. of concentrated HCl and 5 cc. of 56 per cent  $D_2O$  were heated for 100 hours at  $96^\circ$  in a sealed tube. The liquid was distilled off *in vacuo*, and the residue was dissolved in 10 cc. of water and again brought to dryness. This process was repeated four times. The glycine was finally precipitated from alcohol with aniline. The compound contained  $0.92 \pm 0.02$  atom per cent deuterium.

*l(-)-Proline*—980 mg. of substance were dissolved in a mixture of 10 cc. of concentrated HCl and 10 cc. of water containing 50 atom per cent deuterium. The solution was refluxed for 20 hours and the water distilled off *in vacuo*. The residue was dissolved in 30 cc. of  $H_2O$  and again taken to dryness. This was repeated six times in all. The residue was taken up in 30 cc. of  $H_2O$ , brought to pH 6 with  $NaHCO_3$  and evaporated to dryness. The proline was extracted from the residue with hot alcohol, and, on cooling, it crystallized, the yield being increased by addition of ether. The product contained  $0.10 \pm 0.02$  atom per cent deuterium.

*l(-)-Cystine*—6 gm. of cystine were dissolved in a mixture of 9 cc. of concentrated HCl and 9 cc. of 56 per cent  $D_2O$  and heated in a sealed tube at  $98^\circ$  for 5 days. Decomposition was evidenced by the formation of a dark precipitate. The solution was diluted with water, treated with charcoal, and filtered. The filtrate was evaporated to dryness under reduced pressure. The residue was taken up in 50 cc. of  $H_2O$  and again evaporated to dryness. This

was repeated four times in all. The residue was then taken up in 150 cc. of water, decolorized again with charcoal, and neutralized with  $\text{NH}_4\text{OH}$ . The crystals were filtered off, washed with water, and recrystallized twice by dissolving in dilute  $\text{HCl}$  and neutralizing. The product, weighing 4.08 gm., had  $[\alpha]_D = -82^\circ$  in  $\text{N HCl}$ . It contained  $1.51 \pm 0.03$  atom per cent deuterium.

*l(-)-Tyrosine*—5.5 gm. of tyrosine were dissolved in a mixture of 10 cc. of concentrated  $\text{HCl}$  and 10 cc. of 50 per cent  $\text{D}_2\text{O}$  and refluxed for 18 hours. The solvent was distilled off *in vacuo*, and the residue dissolved in 1 liter of dilute  $\text{HCl}$ , decolorized with charcoal, and filtered. The filtrate was brought to pH 6 with  $\text{NaOH}$  and the tyrosine filtered off. Recrystallization was repeated in this way a total of three times. Yield 2.08 gm. It contained  $0.91 \pm 0.05$  atom per cent deuterium.

*Conversion into Dibromotyrosine*—500 mg. of this product were dissolved in dilute  $\text{HCl}$ . Bromine water was added until the solution remained faintly yellow. The solution was evaporated *in vacuo*, and the residue redissolved in water, treated with charcoal, and neutralized with ammonia. The recrystallized dibromotyrosine contained  $0.00 \pm 0.08$  atom per cent deuterium.

*dl-Phenylalanine*—1 gm. of phenylalanine was dissolved in 10 cc. of concentrated  $\text{HCl}$  and 10 cc. of 50 per cent  $\text{D}_2\text{O}$  and refluxed for 18 hours. The solvent was distilled off *in vacuo*, the residue dissolved in 15 cc. of water, and the solution again brought to dryness. 20 cc. of water were added and the solution was neutralized with  $\text{NH}_4\text{OH}$ . 0.40 gm. of substance was crystallized. It contained  $0.04 \pm 0.03$  atom per cent deuterium.

*l(+)-Glutamic Acid*—4 gm. of glutamic acid were kept at  $98^\circ$  for 5 days in a sealed tube with 3.5 cc. of concentrated  $\text{HCl}$  and 3.5 cc. of 56 per cent  $\text{D}_2\text{O}$ . The solution remained clear and colorless. The solution was brought to dryness *in vacuo*, 60 cc. of water were added, and the solution again brought to dryness. This was repeated three times in all. The final residue was taken up in 50 cc. of water, neutralized to Congo red with  $\text{NH}_4\text{OH}$ , and the crystals filtered off and washed. The product was recrystallized twice from 50 cc. of hot water. The glutamic acid contained  $2.01 \pm 0.02$  atom per cent deuterium.



*l*(+)-*Lysine*—2 gm. of lysine monohydrochloride were kept at 98° for 5 days in a sealed tube with 1.5 cc. of concentrated HCl and 1.5 cc. of 56 per cent D<sub>2</sub>O. The water was distilled off, 50 cc. of water were added, and the solution was again taken to dryness. This was repeated four times in all. The residue was dissolved in 10 cc. of water, decolorized with charcoal, and filtered. To the filtrate were added 50 cc. of alcohol. On neutralization with aniline, lysine monohydrochloride crystallized. The crystals were washed with alcohol and recrystallized by dissolving in a small volume of water and adding alcohol. The lysine monohydrochloride contained  $0.00 \pm 0.02$  atom per cent deuterium.

TABLE I

*Lability of Deuterium in Amino Acids Previously Treated with Heavy Water*  
The substances were boiled with 20 per cent HCl.

Compound	Boiling time	Deuterium content
	hrs.	atom per cent
Cystine	0	$1.51 \pm 0.03$
	20	$1.19 \pm 0.02$
	96	$0.50 \pm 0.02$
Glutamic acid	0	$2.01 \pm 0.02$
	20	$0.87 \pm 0.02$
	96	$0.11 \pm 0.02$

*Procedure C. Lability of Deuterium in Amino Acids  
Previously Treated with Heavy Water*

The cystine preparation from Procedure B containing 1.51 atom per cent deuterium and the glutamic acid preparation from Procedure B containing 2.01 atom per cent deuterium were used for these exchange reactions. They were refluxed with a large quantity of 20 per cent HCl and samples taken for isolation and analysis after 20 and 96 hours. The amino acids were recovered by evaporation of the solution under reduced pressure, by redissolving in water, and by neutralizing with NH<sub>3</sub>. The results are given in Table I.

## DISCUSSION

The experiments with synthetic deuterioamino acids (leucine, valine, homocystine, and methionine), as well as those prepared

with concentrated  $D_2SO_4$  (leucine and alanine), clearly demonstrate that there exist carbon-bound hydrogen atoms which are stable and do not exchange under the drastic conditions employed. As was expected by Kinney and Adams (3) and by Patterson and du Vigneaud (4), such amino acids with deuterium in the  $\beta$  and  $\gamma$  positions may be used for biological investigation. In a subsequent paper, it will be shown that histidine and glutamic acid also contain stably bound hydrogen.

A number of amino acids contain, besides the stable hydrogen atoms, some which are semilabile and exchange under conditions of protein hydrolysis. This exchange is very slow. We have not

TABLE II

*Atoms of Semilabile Deuterium Introduced per Molecule of Amino Acid*

Compound	Carbon-bound H atoms	Calculated content of deuterium in solvent	Deuterium content of amino acid found	Deuterium per molecule of amino acids	Carbon-bound H actually exchanged
(1)	(2)	(3)	(4)	(5)	(6)
		<i>atom per cent</i>	<i>atom per cent</i>	<i>atom</i>	<i>per cent</i>
Glycine.....	2	27	0.92	0.17	8.5
Proline.....	7	27	0.10	0.03	0.5
Cystine.....	6	28	1.51	0.65	10.8
Tyrosine.....	7	25	0.91	0.40	5.7
Phenylalanine.....	8	25	0.04	0.02	0.2
Glutamic acid.....	5	26	2.01	0.70	14.0
Lysine hydrochloride....	9	25	0.00	0.00	0.0

been able to introduce into any of the amino acids more semilabile deuterium than corresponds to 1 atom of deuterium per molecule.

In Table II are collected all the data on the amino acids investigated by Procedure B. The deuterium content of the medium at the end of the reaction (Column 3) is calculated from the known amounts of heavy water and concentrated hydrochloric acid employed as well as the small amount of exchangeable hydrogen introduced by the carboxyl and amino groups. Since during the process of isolation all the labile deuterium ( $NH_2$ ,  $COOH$ ,  $OH$ , etc.) has been removed, the values in Column 4 represent

carbon-bound deuterium. If the equilibrium constant for the exchange reaction  $\text{RH}_n + \text{HOD} \rightleftharpoons \text{RH}_{n-1}\text{D} + \text{HOH}$  were known, the deuterium content of any amino acid could be calculated on the basis of the exchange of 1 or more hydrogen atoms. This constant is not known, but it is certainly not far removed from unity (11). Thus for glycine, if 1 hydrogen atom had exchanged, then the deuterium content of the glycine isolated would be 27/5 (the deuterium content of the medium divided by the number of hydrogen atoms in glycine) or 5.4 atom per cent. Since only 0.92 atom per cent deuterium was found, it may be formally stated that  $0.92/5.4 = 0.17$  atom of hydrogen has exchanged. This corresponds to 8.5 per cent of the carbon-bound hydrogen (Column 6).

Similar considerations apply to the other amino acids tested. As will be seen from Column 6, only glycine, cystine, tyrosine, and glutamic acid show definite exchange, for the values represent the percentage of exchange which could be anticipated if all carbon-bound hydrogen was involved.

From the values given in Table II, one can conclude that proline, phenylalanine, and lysine contain practically no semilabile hydrogen. The minute amounts of deuterium which may be present could have been introduced by slight racemization, occurring during the treatment with mineral acid. The high deuterium content of cystine was probably due to this reaction. It thus seems likely that in cystine the exchange has occurred at the  $\alpha$ -carbon atom; in glycine this must have been so.

The deuterium in tyrosine, as mentioned before, was in ortho position to the phenolic group, as it could be removed by bromination. This is furthermore confirmed by the behavior of phenylalanine, which differs from tyrosine only by the absence of the phenolic hydroxyl group and which is resistant to exchange. The mechanism of the exchange of glutamic acid is still obscure. As mentioned before, this may be connected with the known equilibrium existing between this compound and pyrrolidonecarboxylic acid.

All experiments indicate that with the exception of glycine and cystine, the  $\alpha$ -hydrogen atom in amino acids is stable. This is not surprising in view of the fact that, with a few exceptions, they do not appreciably racemize under conditions of acid hydrolysis. Exchange of the  $\alpha$ -hydrogen atom should parallel racemization.

This view is supported by the behavior of our deuteroleucine, which had been prepared by the action of concentrated  $D_2SO_4$ . During the process the substance was racemized. In a subsequent paper it will be shown that concentrated  $D_2SO_4$  leads to an exchange of only the  $\alpha$ -hydrogen atom in higher fatty acids. The racemization of leucine suggests that in amino acids too, this procedure involved the  $\alpha$ -hydrogen atom. The fact that the deuterium thus introduced does not exchange in the presence of more dilute acids (20 per cent  $H_2SO_4$ ) must be taken as an indication of the high stability of the  $\alpha$ -hydrogen atom of this compound.

#### SUMMARY

1. Experiments were carried out on the stability of the hydrogen atoms in amino acids as a preliminary to biological studies with deuterium as an indicator for amino acid metabolism. Two series of exchange reactions were carried out with boiling mineral acids, approximating the conditions of protein hydrolysis.

2. In the first series leucine- $\beta, \gamma-d_2$ , valine- $\beta, \gamma-d_2$ , homocystine- $\beta, \gamma-d_4$ , and deuteroleucine and deuterioalanine with deuterium most probably in the  $\alpha$  position did not lose any deuterium when boiled with mineral acids in ordinary water. Methionine- $\beta, \gamma-d_2$  lost 6 per cent of its original deuterium under these conditions. This was attributed to slight decomposition occurring during the treatment with acids.

3. In the second series a number of ordinary amino acids (glycine, proline, phenylalanine, tyrosine, cystine, glutamic acid, and lysine) were treated with hydrochloric or sulfuric acid in heavy water, and recrystallized from ordinary water. No deuterium was introduced into lysine and only traces into proline and phenylalanine. Small amounts of deuterium (less than 1 atom per molecule) were found in glycine, tyrosine, cystine, and glutamic acid.

4. The deuterium in tyrosine is ortho to the phenolic group. This deuterium is quantitatively removed from the compound by converting it into dibromotyrosine.

5. The introduction of deuterium into cystine is most probably due to racemization, and into glutamic acid to its equilibrium with pyrrolidonecarboxylic acid.

6. Deuterium introduced into amino acids by boiling with acids

in heavy water can be removed by boiling with acids in ordinary water.

7. The stability of the carbon-bound hydrogen in amino acids renders deuterium applicable as an indicator for their metabolism. The presence of semilabile hydrogen should not interfere, as deuterium introduced into these positions can be removed by successive treatments with ordinary water.

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## DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

### XIV. BIOLOGICAL FORMATION OF DEUTEROAMINO ACIDS\*

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In Paper XIII (1), the uptake of deuterium into amino acids when treated *in vitro* with heavy water has been discussed. While most of the carbon-bound hydrogen atoms of these compounds were stable and did not exchange with the deuterium of the medium, there was observed in some a slow exchange. This uptake of deuterium into amino acids is reversible. The deuterium can be removed by subsequent treatment with acids in ordinary water.

In the living organism, there take place other processes in which the hydrogen is involved, such as chemical reactions. If the body fluids of an animal contain heavy water, these processes may lead to the introduction of deuterium into organic linkages. Deuterium introduced by such reactions differs in general from that studied in Paper XIII, as it is not removable by treatment with reagents in ordinary water. Fatty acids and sterols (2) isolated from animals which had drunk heavy water contained deuterium which was resistant to prolonged treatment with alkali or acid in ordinary water.

In this paper, we report on similar experiments in which amino acids were isolated from mice which had received heavy water to drink. Ussing (3) has already demonstrated an uptake of deuterium into proteins of rats kept under similar conditions. This uptake was larger with the protein of such muscles which were

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stimulated than with resting muscles, and was still lower with the skin. Recently, Krogh and Ussing (4) found deuterium present even after hydrolysis of the protein. They suggest that the deuterium introduced was stably bound. Similar experiments carried out by Günther and Bonhoeffer (5) on yeast grown in heavy water have indicated the presence of deuterium in the total amino acids after the hydrolysis of the yeast protein.

While our work was in progress, Stekol and Hamill (6) published data on the deuterium content of total protein and of tyrosine and cystine isolated from mice given heavy water to drink. The deuterium content of all these substances was very high when compared with that of the water in the body fluids of the animals.

We have not analyzed the deuterium content of total proteins or the total hydrolysate because of the extreme difficulty in drying such amorphous material sufficiently for deuterium analysis and of interpreting analytical results from such complex material as proteins or amino acid mixtures. We have, therefore, confined ourselves to the investigation of pure amino acids.

Two groups of mice, one on a bread diet and the other on a casein diet, were kept on heavy water for various lengths of time, and from their carcasses were isolated nine pure amino acids in one case, and ten in the other. With the exception of lysine, all contained considerable amounts of deuterium which had not been removed during the prolonged treatment with 20 per cent HCl during the hydrolysis. The deuterium content of these amino acids was in general higher than that introduced into the amino acids by exchange from heavy water, as described in Paper XIII (1). The deuterium biologically introduced must be in positions different from those into which it is introduced by treating the amino acids *in vitro* with heavy water. This could be proved in the cases of glutamic acid and histidine in which, on subsequent treatment with boiling 20 per cent HCl, the deuterium content remained constant.

#### EXPERIMENTAL

The body fluids of the animals were raised to a heavy water concentration of about 1.5 per cent at the beginning of the experiment by injecting the calculated amount of concentrated D<sub>2</sub>O. The animals then received, instead of ordinary water, 2.34 per cent heavy water (2). The deuterium content of the body fluids

was thus kept constant at about 1.5 atom per cent throughout the experiment, and all reactions in the bodies of the animals went on in a medium having this composition. Two sets of mice on different diets were investigated.

*Group 1*—Besides the heavy water, the mice received dried whole wheat bread *ad libitum*. Three mice of 18 gm. each were kept for 19 days and four mice of 25 gm. each were kept for 98 days on this diet. The body fluids of the animals at the end of the experiments contained 1.62 and 1.48 atom per cent deuterium respectively. The carcasses of the animals were pooled for the isolation of the amino acids.

*Group 2*—Twenty mice of 18 to 20 gm. in weight were treated with heavy water as above. The diet, which was the same as that used by Stekol and Hamill (6), consisted of 70 per cent rolled oats, 15 per cent of powdered whole milk, and 15 per cent yeast and contained 7 per cent of water. The only difference between our experiments and those of the above authors is that we injected the mice with heavy water at the start of the experimental period. The animals were maintained on this diet for 10 days. The deuterium content of the body fluids at the end of the experiment was 1.60 atom per cent.

#### *Isolation of Amino Acids from Carcasses*

The animals were killed and the gastrointestinal tract was removed and discarded. The water was distilled off *in vacuo* from the pooled carcasses for the determination of the deuterium content of the body fluids. The carcasses were then continuously extracted with hot acetone for 8 hours and with ether for 7 hours. The defatted material was hydrolyzed by boiling 20 hours with 10 times its weight of 20 per cent HCl. The solution was concentrated to a thick syrup under reduced pressure and the distillation was continued with the slow addition of water in order to remove HCl as completely as possible.

*Tyrosine* and *cystine* were obtained by isoelectric precipitation and separated by fractional crystallization. More *cystine* was obtained from mother liquors by precipitation as cuprous mercaptide.

*Arginine*, *histidine*, and *lysine* were isolated as flavianate, mercuric chloride complex, and phosphotungstate respectively. Each



*Glutamic* and *aspartic acids* were obtained by precipitation with  $\text{Ba}(\text{OH})_2$  and alcohol. They were prepared as the free acids, glutamic acid through the hydrochloride and aspartic acid by way of the copper salt.

*Leucine* was isolated from the copper salt fraction which was insoluble in both hot water and hot methyl alcohol.

*Proline* and *glycine* were obtained by the methods of Bergmann (7) and Bergmann and Fox (8). All the compounds isolated were recrystallized. Their analyses are shown in Table I.

The deuterium content of the amino acids is given in Table II, Columns 3 and 4. The values given in Columns 5 and 6 are cal-

TABLE I  
*Analyses of Amino Acids Isolated from Carcasses*

Compound	Found		Calculated
	Group 1	Group 2	
Cystine, %.....		S 26.9	26.7
Tyrosine, %.....	N 7.8		7.7
Arginine hydrochloride, %.....	" 26.2	N 26.4	26.6
Histidine " %.....	" 19.3	" 19.9	20.05
Lysine " %.....	" 15.2	" 15.4	15.4
Glutamic acid, %.....	" 9.5	" 9.5	9.5
Aspartic " %.....	" 10.3	" 10.4	10.5
Proline, %.....	" 12.1	" 12.1	12.2
Glycine as hippuric acid, °C.....	M.p. 188	M.p. 188	
Leucine, %.....	N 10.4	N 10.5	10.7
" $[\alpha]_D$ in 20% HCl, degrees..	16.6	18.6	

culated in a manner analogous to that in Table II of Paper XIII (1). The medium in which the reactions took place consisted of 1.5 per cent heavy water. Columns 7 and 8, as in the preceding paper, represent the percentage of "exchange" if all carbon-bound hydrogen atoms were involved. The value, in other words, is the percentage of the maximal deuterium uptake possible.

*Stability of Deuterium in Amino Acids Isolated from Mice*

*Histidine*—0.706 gm. of histidine monohydrochloride from Group 2 was boiled under a reflux with 20 per cent HCl in ordinary

water for 108 hours. The optical rotation of the solution did not change (observed in a 2 dm. tube,  $+0.50^\circ$ ). The solution was evaporated to dryness under reduced pressure, the residue dissolved in 95 per cent ethanol, and the histidine monohydrochloride precipitated by the addition of aniline. It was recrystallized from water by the addition of alcohol. 0.549 gm. of substance was obtained. The deuterium content before treatment was  $0.20 \pm 0.02$  and after treatment  $0.19 \pm 0.02$  atom per cent.

TABLE II  
*Biological Uptake of Deuterium into Amino Acids*

(1)	Carbon-bound H atoms (2)	Deuterium content of amino acids		Deuterium per molecule of amino acid		Carbon- bound H actually exchanged	
		Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
		(3)	(4)	(5)	(6)	(7)	(8)
		atom per cent	atom per cent	atoms	atoms	per cent	per cent
Glycine.....	2	$0.14 \pm 0.02$	$0.15 \pm 0.02$	0.5	0.5	23	23
Leucine.....	10	$0.12 \pm 0.02$	$0.08 \pm 0.01$	1.0	0.7	10	7
Lysine HCl.....	9	$0.00 \pm 0.02$	$0.00 \pm 0.02$	0.0	0.0	0	0
Tyrosine.....	7	$0.05 \pm 0.03$	$0.08 \pm 0.02$	0.4	0.6	5	7
Proline.....	7	$0.08 \pm 0.04$	$0.16 \pm 0.02$	0.5	1.0	7	14
Arginine HCl....	7	$0.13 \pm 0.02$	$0.06 \pm 0.02$	1.3	0.6	19	9
Histidine " ....	5	$0.24 \pm 0.02$	$0.20 \pm 0.02$	1.6	1.3	32	26
Glutamic acid...	5	$0.36 \pm 0.02$	$0.37 \pm 0.01$	2.2	2.2	44	44
Aspartic " ....	3	$0.18 \pm 0.02$	$0.24 \pm 0.04$	0.9	1.1	30	37
Cystine.....	6		$0.09 \pm 0.03$		0.7		12

*Glutamic Acid*—0.360 gm. of glutamic acid from Group 1 was refluxed with 25 cc. of 20 per cent HCl in ordinary water for 108 hours. The optical rotation of the solution did not change (observed in a 2 dm. tube,  $+0.96^\circ$ ). The substance was isolated as above and recrystallized from water and alcohol. 0.283 gm. of glutamic acid was obtained. The deuterium content before treatment was  $0.36 \pm 0.02$  and after treatment  $0.38 \pm 0.02$  atom per cent.

#### DISCUSSION

The values given in Table II represent the deuterium which has been introduced *in vivo* from the heavy water into the amino

acids. With the exception of lysine all contained deuterium. Lysine seems to occupy an exceptional position among the amino acids. The amounts of deuterium in the other amino acids are generally higher than that introduced by boiling the compounds with mineral acids in heavy water as described in Paper XIII (1). The amino acids isolated from mice were subjected during the hydrolysis to acid treatment at elevated temperature and some deuterium which might have been present at semilabile positions must have been removed by this procedure. The isotope content in at least some of the amino acids was probably higher in the animal than it was after isolation.

The deuterium in glutamic acid and histidine obtained from the animals was not semilabile, as subsequent treatment with hydrochloric acid did not reduce the original deuterium content. The isotope in the glutamic acid preparation thus must have been in positions different from those into which it is introduced by treatment with heavy water. In the histidine preparation also, the deuterium was stably bound and not removable. According to Hamill (9), 5 out of the 9 hydrogen atoms in histidine exchange rapidly. The deuterium biologically introduced cannot have been one of these.

A process must have been responsible for the biological introduction of this deuterium, which is in principle different from a mere exchange reaction of labile or semilabile hydrogen. It is probable that in other amino acids isolated from the mice the deuterium found was also present in stable positions. The amount of the other amino acids obtained was not sufficient for a treatment similar to that carried out with glutamic acid and histidine.

In the case of glycine, it is certain that the deuterium found even after hydrolysis was semilabile. Glycine contains besides the ionizable hydrogen atoms at the COOH and NH<sub>2</sub> groups only semilabile hydrogen; namely, that of the CH<sub>2</sub> group. The isotope in the glycine preparation isolated from the animal must, therefore, represent semilabile deuterium which was not removed during the hydrolysis of the protein and the subsequent hydrolysis of the hippuric acid employed in the purification. The deuterium content of the glycine in the animal was undoubtedly higher than after its isolation. The fact that a considerable amount of deuterium was still carried through all these processes can be taken

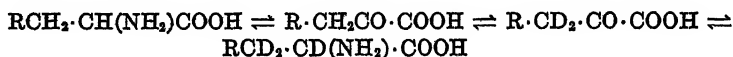
as another indication that the exchange of the carbon-bound hydrogen in glycine is a rather slow process. In Paper XIII, it was shown that glycine when treated for 100 hours with mineral acid at 96° had exchanged only a small fraction—8 per cent—of its carbon-bound hydrogen, while the material from the animal still contained 23 per cent carbon-bound deuterium. It is highly probable that in the animal organism the large quantity of deuterium in glycine had also originated by some process other than mere exchange.

There are two such mechanisms by which deuterium might have been introduced into amino acids *in vivo*; namely, chemical reactions or the action of enzymes which labilize carbon-bound and stable hydrogen without further changing the amino acid. Stekol and Hamill (6) have claimed that proteolytic enzymes can carry out such a labilization. Their results, however, could not be confirmed (10). The fact that the lysine isolated from our animals did not contain deuterium can be taken as further indication against their view. Our lysine was isolated from proteins and must have been subjected to proteolytic enzymes in the animals at least during the absorption of dietary proteins.

In previous papers, the possible occurrence of other enzymes which may labilize carbon-bound hydrogen has been discussed (2). If a substance, isolated from an animal kept on heavy water, contains stable deuterium, it is in many cases difficult to exclude the possibility that the isotope was introduced by such an enzymatic mechanism. The proof for the absence of such reactions has as yet been given only for those compounds which also could be isolated in the deuterium-free state from organisms kept on heavy water. In the case of fatty acids and sterols, it was found that these compounds took up deuterium from the body fluids of adult animals, but they could be isolated in a deuterium-free state from chicks which had developed in a medium of heavy water. The present experiments furnish proof of the absence in mice of enzymes capable of labilizing carbon-bound hydrogen of lysine. This compound, isolated from both series of experiments, did not contain a trace of deuterium. This weakens the probability that such enzymes exist which act on other amino acids. However, proof is still lacking.

The absence of deuterium in lysine, furthermore, proves that

no chemical reaction which involves the carbon chain had occurred on this amino acid in the animal. This finding is in good agreement with the studies on the indispensability of this amino acid. On the other hand, the histidine, which is also known to be an indispensable amino acid, contained stably bound deuterium. The indispensability of histidine does not exclude the possibility that the deuterium had a source in a chemical reaction. It is well known that it can be substituted in the diet of growing animals by the corresponding  $\alpha$ -hydroxy or  $\alpha$ -keto acid, or even by its optical antipode (11), so the animal must be able to amidize the  $\alpha$ -hydroxy or  $\alpha$ -keto acids. If the reaction is carried out in a medium of heavy water, a minimum of 1 and a maximum of 3 stable deuterium atoms can be introduced, for the ketone group, by enolization, may labilize the hydrogen of the  $\beta$ -carbon atom.



The deuterium content of histidine and of other amino acids may well be due to such or similar reactions, while the absence of deuterium in lysine indicates that they have not occurred with this compound. This is well in accord with the observation of Berg (12) that lysine, in contrast to histidine, is not efficient for growth when given as its unnatural optical isomer. The organism does not seem to have the ability of amidizing the  $\alpha$ -keto acid corresponding to lysine.

Leucine, another indispensable acid isolated from the mice, also contained deuterium. It was shown in Paper XIII that all the carbon-bound hydrogen atoms in leucine are stably bound. The deuterium in the substance isolated from mice must have been stable. Our leucine preparation, however, was not separated into the isomers (norleucine, isoleucine, and leucine), and we are not in a position to state in which of them the deuterium is present. Leucine and isoleucine, as shown by Rose (13), can be replaced for growth experiments by the corresponding  $\alpha$ -hydroxy and  $\alpha$ -keto acids, and the possibility exists that the deuterium in our experiments was introduced by successive deamidation and amidation.

The biological uptake of deuterium into fatty acids and sterols in mice was taken as indicating fatty acid or sterol synthesis (2).

Such a reasoning is not permissible in work with amino acids, as the term "amino acid synthesis" is not yet well defined. While the formation of alanine from pyruvic acid is generally considered to be a synthesis, the amidation of other, more complex, keto acids is not usually so regarded. In biological work, it is customary to classify amino acids from the point of view of whether they are dispensable or indispensable. The indispensability does not exclude the possibility that chemical reactions may go on in the compound after its absorption. The deuterium technique cannot give any indication as to dispensability or indispensability, but may give information as to whether or not chemical reactions have occurred.

An indispensable amino acid is defined by Rose (14) as "one which cannot be synthesized by the animal organism... at a speed commensurate with the demands for normal growth." Indispensability thus does not mean that the animal lacks all ability to form the amino acid in question from other food constituents. Arginine, which, according to this definition, is indispensable, can be formed in the animal to some extent, but not in sufficient amounts (14). The large quantities of deuterium (almost 2 atoms) in the arginine sample isolated from Group 1 may be connected either with the new formation of the ornithine moiety or with the other reactions discussed above. None of our findings is in disagreement with those obtained in studies of indispensability.

#### SUMMARY

1. The deuterium content of the body fluids of two groups of mice was raised to 1.5 atom per cent and kept for average periods of 63 and 10 days respectively. Group 1 obtained a diet of bread and Group 2 of casein. The carcasses of the mice were hydrolyzed with 20 per cent HCl and nine different amino acids were isolated from Group 1 and ten from Group 2. The amino acids isolated were glycine, leucine, lysine, tyrosine, proline, arginine, histidine, glutamic acid, aspartic acid, and cystine.

2. With the exception of lysine all these amino acids contained carbon-bound deuterium. The amount of deuterium was generally higher than that introduced into amino acids *in vitro* by exchange with heavy water at elevated temperature.

3. The deuterium in the amino acids isolated from mice is relatively stably bound, as it was not removed during hydrolysis of the protein with acid. In the case of histidine and glutamic acid, the deuterium was not "semilabile," for when these compounds were subsequently boiled with 20 per cent HCl for 108 hours, their deuterium content did not decrease.

4. It was concluded that this deuterium was not introduced by a mere exchange reaction with the heavy water of the body fluids with which the amino acids were in contact. Two mechanisms are discussed which might have led to the formation of the deuterio compounds: (1) chemical reactions at the carbon chain, such as synthesis or deamidation and reamidation of the resulting keto acid; (2) enzymatic reactions, by which carbon-bound hydrogen is labilized without further changes of the molecule.

5. Neither one of these two reactions can have occurred in the case of lysine, as both should have led to the formation of deuterio-lysine. This compound seems to occupy an exceptional position among the amino acids investigated.

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# DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

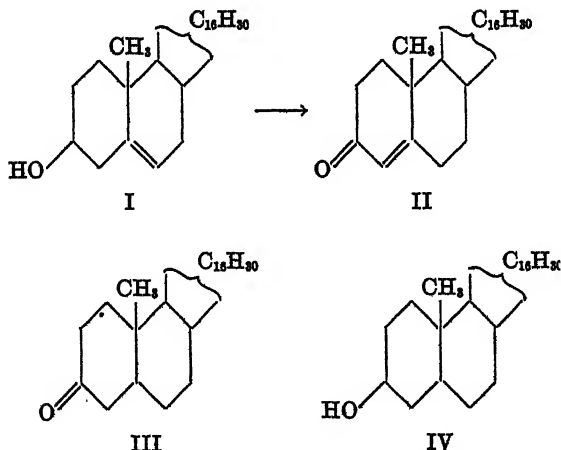
## XV. FURTHER STUDIES IN COPROSTEROL FORMATION. THE USE OF COMPOUNDS CONTAINING LABILE DEUTERIUM FOR BIOLOGICAL EXPERIMENTS\*

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(Received for publication, May 12, 1938)

In a previous publication by Schoenheimer, Rittenberg, and Graff (1) a theory was advanced according to which the biological conversion of cholesterol (I) to coprosterol (IV) does not go by direct hydrogenation of the double bond but via cholestenone (II) and coprostanone (III).



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† This report is from a dissertation submitted by M. Anchel in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

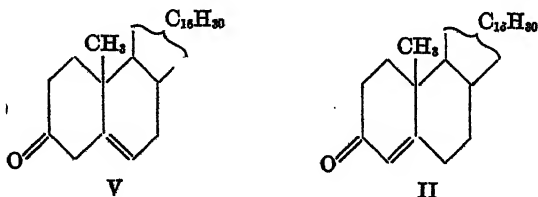


If the two ketones were actually intermediate steps, both of them should be easily converted to coprosterol when fed. Cholestenone administered to dogs was found to give rise to increased excretion of coprosterol (1). At about the same time, similar results were obtained independently by Rosenheim and Webster (2), who also suggest cholestenone as an intermediate in coprosterol formation. The direct conversion of coprostanone to coprosterol was demonstrated (1) by administering coprostanone-4,5- $d_2$  to a dog and to a human being, and isolating deuterocoprosterol from the stools. While the biological conversion of coprostanone to coprosterol had thus been definitely proved by the application of isotopes, the conversion of cholestenone to coprosterol had been demonstrated only by balance experimentation.

Indications exist in the literature that a number of substances which are not chemically related to sterols, when given in sufficiently large quantities, may induce either sterol formation from other compounds, or increased sterol excretion. Thus, Eckstein and Treadwell (3) have shown that feeding of fat leads to increased sterol formation, and Channon and Tristram (4) have found that several unsaponifiable substances lead to increased sterol excretion. The results of the earlier experiments in which feeding of cholestenone led to an increase in sterol excretion may, therefore, not necessarily indicate a conversion of cholestenone to coprosterol, but may be interpreted as an increase in sterol excretion induced by the cholestenone fed.

At the time of the first publication (1) deuterio- $\Delta^4$ -cholestenone was not available. We have now administered this compound to a human being, and subsequently isolated deuterocoprosterol from the stools.

The method of introducing deuterium into cholestenone by hydrogenation of a double bond is not feasible. The preparation of deuterio- $\Delta^4$ -cholestenone was carried out by rearrangement of  $\Delta^5$ -cholestenone (V) to (II) in an alcohol- $D_2O$  mixture containing alkali.



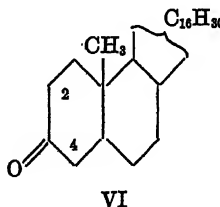
It was expected that in this reaction 4 hydrogen atoms would exchange with those of the medium. 3 of these at carbon atoms 2 and 4 would originate from enolization of the ketone group in the alkaline medium. Those at carbon atom 2 would be labile. The deuterium atoms introduced at carbon atom 6 by a shift of the double bond might be stably bound, as might also be that at carbon atom 4.

The reaction actually led to deuteriocholestenone with about 4 deuterium atoms. All of these, however, proved to be labile on treatment with hot alcoholic alkali. The complete lability of the deuterium in the compound may suggest that in alkaline solution an equilibrium exists between the two unsaturated ketones, the double bond shifting also from Ring A to Ring B. The rate of exchange must have been slow, since the original material was recrystallized from absolute alcohol before analysis. This was to be expected, since the hydrogen exchange is probably connected with an equilibrium of tautomeric forms. The rate of such exchanges is in general of a much smaller order than those of the hydrogen atoms of carboxyl, hydroxyl, etc.

Despite the complete lability of the deuterium atoms in the presence of alkali, the compound proved to be suitable for our purpose. After the material containing labile deuterium had been fed, the coprosterol isolated from the stools contained stably bound deuterium; *i.e.*, not removable by treatment with hot alkali. The biological reduction of the double bond and of the ketone group had thus stabilized the deuterium. In contrast to the ketone, coprosterol contains only 1 labile hydrogen atom; namely, that of the newly formed hydroxyl group.

Since the experiment with cholestenone suggested that under certain conditions compounds containing labile deuterium could be used in metabolism studies, a second experiment was carried out in which another compound containing labile deuterium was administered. In the previous publication (1) coprostanone-4,5- $d_2$  was fed in which the deuterium at carbon atom 5 was stable, while that at carbon atom 4 was labile and could be removed by treatment with alkali. We have now administered coprostanone containing only labile deuterium, prepared by treatment of ordinary coprostanone with alkali and  $D_2O$ . The deuterium in the compound must have been introduced by enoli-

zation of the ketone group, and therefore must be attached to carbon atoms 2 or 4 (VI). After the administration of this



material, the coprosterol of the stools contained stably bound deuterium.

In the previous experiment (1) in which cholestenone was fed to a dog, on a diet on which very little coprosterol was formed, cholesterol excretion was increased, suggesting that cholestenone could also be converted into cholesterol. Diels (5) has studied this question further, and has reported an increase of total sterols in the livers of guinea pigs after the administration of cholestenone. As the melting point of the sterol isolated by him was lower than that of the sterols isolated from controls, he has suggested that besides cholesterol another sterol was formed. In an experiment on the conversion of cholestenone to cholesterol we have fed to mice the same deuterio- $\Delta^4$ -cholestenone as described above. The cholesterol isolated from the animals and purified with digitonin and via the dibromide contained too insignificant an amount of deuterium to be taken as a proof of its origin from the administered cholestenone. This negative result may be due to one of two reasons: the ketone may not have been converted to cholesterol, or the deuterium may have been lost during the conversion process.

#### EXPERIMENTAL

*Preparation of Deutero- $\Delta^4$ -Cholestenone*—500 mg. of  $\Delta^4$ -cholestenone, m.p. 127°, prepared according to the method of Butenandt and Schmidt-Thomé (6), were refluxed for 35 minutes in a solution of 7.60 cc. of absolute ethyl alcohol, 2.45 cc. of 80 per cent D<sub>2</sub>O, and 80 mg. of NaOH. The solution was cooled in an ice-salt mixture, and the crystals filtered off. The alkali was removed by washing with water containing the same concentration of deuterium atoms as were labile in the alcoholic solution in which the

substance had been prepared. As it was later found that washing with ordinary water did not remove appreciable amounts of deuterium, in subsequent preparations the material for feeding experiments was washed with water. 410 mg. of cholestenone were obtained, m.p. 79–80°. 150 mg. were recrystallized from 0.5 cc. of absolute alcohol, yielding 124 mg. of melting point 81°, and containing 4.75 atom per cent deuterium. The theoretical amount of deuterium calculated for an exchange of 4 hydrogen atoms is 4.91 atom per cent, on the assumption that the deuterium atoms are distributed in a random manner.

The remaining 260 mg. were recrystallized twice, each time from 31 cc. of a mixture of 75 per cent alcohol, and 25 per cent water containing 2 per cent NaOH. The material recovered was recrystallized from absolute alcohol, yielding 200 mg. of material melting at 78–80° and containing 0.19 atom per cent deuterium. The deuterium content had thus been reduced by treatment with alkali to about 4 per cent of its original value.

*Fractionation of Coprosterol-Dihydrocholesterol Mixture*—Human feces contain, besides coprosterol, smaller amounts of dihydrocholesterol. In the experiments in which deuteriocholestenone was fed, fractionation of the sterol mixture was essential, in order to be sure that the isotope was actually present in the coprosterol. The usual procedure for fractionating coprosterol and dihydrocholesterol by epimerizing the fecal sterols and isolating epicoprosterol (7) was not employed, as this reaction might have led to a loss of deuterium.

For the purification of coprosterol in the experiment described below we have made use of the difference in behavior of coprosterol digitonide and dihydrocholesterol digitonide toward organic solvents. Coprosterol has been reported to be more easily extracted with ether from its digitonide than dihydrocholesterol (8). We have now found that this is also true when benzene is used, the extraction in this case being more rapid than with ether. This fractionation was investigated first with a mixture of the pure compounds, deuterium being used as an indicator.

600 mg. of synthetic coprosterol-3,4,5- $d_3$  (m.p. 100–101°, containing  $2.32 \pm 0.05$  atom per cent deuterium)<sup>1</sup> and 600 mg. of

<sup>1</sup> The preparation of this compound will be described in a later publication.

ordinary dihydrocholesterol were dissolved together in 50 cc. of 80 per cent alcohol, and precipitated with 450 cc. of a 1 per cent solution of digitonin in 80 per cent alcohol. 4.6 gm. of the digitonide were extracted for 5 hours in a thimble suspended over boiling benzene. 91 mg. of sterol were obtained and recrystallized from methyl alcohol; 80 mg. of material containing  $1.75 \pm 0.04$  atom per cent deuterium were finally obtained, indicating that the extract consisted of a mixture of 75 per cent of coprosterol and 25 per cent of dihydrocholesterol.

*Conversion of Deuterocholestenone to Deuterocoprosterol  
by Human Being*

3 gm. of a preparation of deutero- $\Delta^4$ -cholestenone containing 3.1 atom per cent deuterium were dissolved in butter and administered in 1 gm. portions in an ordinary diet over a period of 6 hours to a healthy male adult of 28 years. Before and after the meal grapes were taken, the seeds of which served as markers in the stools. The stools were dried with  $\text{Na}_2\text{SO}_4$ , ground, and extracted with ether. The ether residue was saponified with methyl alcoholic KOH. The unsaponifiable material, 4.97 gm., contained 66 per cent of sterols. 1.4 gm. of the unsaponifiable material were treated with digitonin, and the digitonide split with pyridine and ether. The crude sterols obtained were recrystallized once from methyl alcohol, yielding 732 mg. of material melting at  $93-95^\circ$  and containing 0.61 atom per cent deuterium.

*Fractionation of Fecal Sterols*—The remainder of the unsaponifiable material from the human feces was treated with digitonin. The digitonide was powdered and extracted with ether in a Soxhlet apparatus for 45 minutes. 164 mg. of partially oily material were removed by this procedure. The rest of the digitonide was powdered again, and extracted for three periods of 5, 7, and 7 hours with hot benzene. The yields were 230, 280, and 280 mg. respectively. After several recrystallizations from methyl alcohol, they were pooled, and 270 mg. of crystals melting at  $99-100^\circ$  (softening at  $95^\circ$ ) were obtained. They contained  $0.73 \pm 0.02$  atom per cent deuterium.

The digitonide remaining in the thimble was thoroughly washed with 80 per cent alcohol to remove excess digitonin, and split with pyridine. 961 mg. of sterol were obtained, which were recrystallized from methyl alcohol, yielding 815 mg. of melting

point 99° (softened at 90°), containing  $0.71 \pm 0.02$  atom per cent deuterium.

The melting point of coprosterol isolated from feces cannot be taken as an accurate measure of its purity (9). The sterol obtained by the benzene extraction of digitonides may contain small amounts of dihydrocholesterol, but the possibility that the deuterium content of the material is due to this substance can be ruled out with certainty as the benzene extraction was found to be effective in the fractionation of the isomers. The fact that the extracted sterol contained as high a proportion of deuterium as that remaining behind serves as proof for the presence of deuterium in the coprosterol. The melting point and deuterium content of both the sterol extracted and that remaining in the thimble were higher than those of the unpurified sterol fraction. This is probably due to the removal by the preliminary ether extraction of oily material which had been adsorbed on the digitonide and had not been removed by simply washing. No attempt was made to isolate dihydrocholesterol. We therefore cannot exclude the possibility that some of the deuterium in this mixture is due to deuterodihydrocholesterol.

The deuterium content of the coprosterol is about one-quarter of that of the cholestenone fed. As a large part of the coprosterol must have come from other sources, the loss of deuterium cannot have been great, and a considerable part of the cholestenone must have been converted.

#### *Conversion of Coprostanone to Coprosterol by Human Being*

2 gm. of deuterocoprostanone having a melting point of 60–62° and a deuterium content of 1.28 atom per cent were prepared by refluxing coprostanone in an alcoholic D<sub>2</sub>O solution containing NaOH. The material was administered to a human being, in two portions of 1 gm. each at an interval of 3 hours. The isolation of the unsaponifiable material from the stools was carried out as above.

1.41 gm. of the total unsaponifiable material were precipitated with digitonin. The digitonide yielded 1.0 gm. of sterol, which was recrystallized from methyl alcohol. 872 mg. of crystals were obtained, melting at 96° (softening at 80°) and containing 0.17 atom per cent deuterium.

experiment was to determine whether the labile deuterium of the ketone would be stabilized by the conversion into sterols. The deuterium content of the sterols was more than 15 per cent of that of the administered ketone.

#### *Feeding Deuterocholestenone to Mice*

2 gm. of deuterocholestenone containing 5.7 atom per cent deuterium were dissolved in lard, mixed with dried ground rye bread, and fed to ten mice over a period of 10 days. The mice were then killed, the intestinal tracts were removed, and the carcasses heated with ethyl alcoholic KOH. The unsaponifiable fraction was treated with digitonin. The digitonide was split with pyridine and ether, yielding 267 mg. of sterol. The dibromide was prepared in the usual manner, recrystallized from ethyl alcohol, and debrominated with zinc in ethyl alcohol. The recovered sterol was recrystallized twice from 90 per cent alcohol, yielding 107 mg. melting at 147–148° and containing 0.08 atom per cent deuterium.

#### SUMMARY

1. Additional support is offered for the theory that cholestenone and coprostanone act as intermediates in the biological formation of coprosterol.

2. The administration of deuterocholestenone to a human being was followed by an excretion of deuterocoprosterol in the stools. The deuterium of the cholestenone fed was labile in that it could be removed by treatment with alkali, while that of the coprosterol isolated was stably bound; *i.e.*, not removable by treatment with alkali.

3. A similar experiment was carried out with coprostanone, containing labile deuterium, with the same result.

4. The cholesterol of mice given deuterocholestenone contained insignificant amounts of deuterium. No conclusions can be drawn from this experiment as to whether or not conversion of cholestenone to cholesterol had occurred, since the deuterium may have been removed during the conversion process.

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## THE MOLECULAR WEIGHT OF CRYSTALLINE CATALASE\*

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(Received for publication, May 31, 1938)

In 1933 Stern (2) used the diffusion method of Northrop and Anson (3) to determine the molecular weights of purified catalase from horse liver, pig liver, cow liver, horse kidney, and cow erythrocytes. He found the average value for the molecular weight of catalase to be 68,900, and stated that the catalase molecule has a dimension similar to that of hemoglobin.

In 1938 Stern and Wyckoff (4) purified horse liver catalase by adsorption and elution and obtained preparations of a *Kat. f.* of 4000 to 9000. Using the analytical ultracentrifuge, they observed three components to be present and were able to separate one of these believed to be identical with catalase. It had a *Kat. f.* of 8500 to 33,400 and a sedimentation constant of  $s_{20} = 11 \times 10^{-13}$ . Stern and Wyckoff estimate the molecular weight as lying between 250,000 and 300,000. They state further that they have found the sedimentation constant of a nearly pure catalase preparation from beef liver to be  $s_{20} = 12 \times 10^{-13}$  but do not state whether this beef liver catalase was prepared according to the directions of Sumner and Dounce for the preparation of crystalline catalase (5).

We have prepared crystalline cow liver catalase, following the Sumner and Dounce method, as described below: Five 300 gm. portions of well ground cow liver were stirred with five 400 cc. portions of 35 per cent dioxane. The material was filtered at room temperature overnight. To the filtrate, amounting to 1750 cc., we added with stirring 340 cc. of dioxane and filtered the material again in a room at 6°. To the 1780 cc. of filtrate we added with

\* A preliminary article describing this determination has been published (1).

stirring 183 cc. of dioxane and filtered the mixture again in the cold. The precipitate was scraped from the filter paper, stirred up with 50 cc. of water and 2 cc. of saliva, and filtered for 2 hours at room temperature, and then overnight in the cold room. The filtrate, which contained some catalase crystals mixed with protein impurity, was centrifuged clear. To the clear supernatant solution we added saturated ammonium sulfate slowly and with cooling. After several hours the crystals of catalase were centrifuged off in the cold room. It was necessary to centrifuge 2 hours at 2500 R.P.M. to precipitate the crystals completely. The supernatant was discarded, and the catalase crystals were dissolved in 30 cc. of water containing a few drops of phosphate buffer of pH 7.4. The solution of catalase was centrifuged clear and the supernatant solution transferred to another 50 cc. centrifuge tube.

TABLE I  
*Centrifugation at 65,000 Revolutions per Minute*

pH	$s_{20} \times 10^{13}$
6.3	11.2
6.8	11.3
8.6	11.2
9.6	11.4
Mean value.....	11.3

To this solution we added saturated ammonium sulfate and acid potassium phosphate cautiously and with cooling. After 3 hours, the resulting crystals of catalase were centrifuged off and dissolved in a solution composed of 5 per cent NaCl and 0.64 per cent phosphate buffer. The solution contained approximately 1 per cent of catalase.

The sedimentation constant was determined as described by Svedberg (6). Since our catalase solutions were too deeply colored to permit use of the scale method, the centrifugations were carried out by the light absorption method, with monochromatic light of wave-length 546 m $\mu$ . The cell thickness was 12 mm. Table I gives the results of centrifugations at 65,000 R.P.M. (centrifugal force 310,000 times gravity) with phosphate buffers at various pH values.

In the four experiments of Table I the catalase was found to be a homogeneous substance, very slightly contaminated with impurity. Fig. 1 shows sedimentation curves for catalase. At pH 9.9 it was split into smaller molecules. Two different components with  $s_{20} = 1.2 \times 10^{-13}$  and  $s_{20} = 4.5 \times 10^{-13}$  could be distinguished. When the catalase solution was dialyzed against an acid buffer, it was first precipitated (around the isoelectric point), but after some time (the solution getting more acid) it

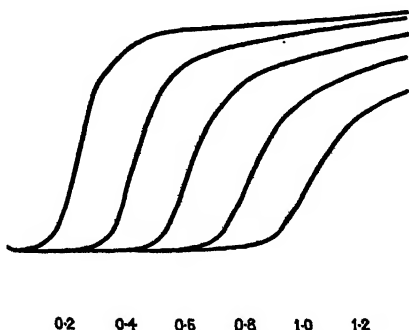


FIG. 1. Sedimentation curves for crystalline catalase at pH 8.6. Centrifugal force 310,000 times gravity. Difference between the exposures 10 minutes;  $s_{20} = 11.2 \times 10^{-13}$ . The ordinate represents the relative concentration; the abscissa, the distance from the meniscus in cm.

partly redissolved. The following centrifugations were made in acetate buffers after dialysis.

pH	$s_{20} \times 10^{13}$
2.8	1.6 Rather homogeneous
3.2	13.1 Inhomogeneous

The diffusion constants were measured in the steel cell described by Lamm (7). Here 1 per cent of catalase was present in 5 per cent NaCl, 0.46 per cent  $\text{Na}_2\text{HPO}_4$ , and 0.18 per cent  $\text{KH}_2\text{PO}_4$ . The diffusion curves were found to be almost ideal distribution curves, indicating a homogeneous substance. Here  $D_{20} = 4.1 \times 10^{-7}$  sq. cm. per second. Stern (2) found the value

$D = 7.79 \times 10^{-7}$  sq. cm. per second, but the method used by us is undoubtedly much more accurate. Our value is also in good agreement with diffusion constants determined for other proteins with sedimentation constants around the value found for catalase (compare the table in Svedberg (6)).

The partial specific volume of catalase was determined by weighing a known volume of the solution and of the sodium chloride-phosphate solvent. It was found to be 0.73 ml. per gm.

### *Molecular Weight of Catalase*

Using the formula  $M = RTs_{20}/D_{20} (1 - V\rho)$  in which  $M$  represents molecular weight,  $V$  partial specific volume, and  $\rho$  density of water at  $20^\circ$ , we have calculated the molecular weight for crystalline beef liver catalase to be 248,000,<sup>1</sup> which is nearly 4-fold the value for horse hemoglobin (6). Sumner and Dounce (5) have found the iron content of crystalline catalase to be approximately 0.1 per cent or about one-fourth that of hemoglobin. It is, therefore, apparent that crystalline catalase must contain 4 atoms of iron, or the same number as hemoglobin.

The authors want to express their thanks to Professor The Svedberg for the privilege of working in his laboratory and for his kind interest. The expenses for the work were defrayed by grants from the Guggenheim, the Andersson, and the Nobel Foundations.

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<sup>1</sup> In our preliminary paper (1) we erroneously reported the molecular weight of catalase to be 263,000 because the distance from the center of rotation of the ultracentrifuge to the index had been wrongly taken, making the value for the sedimentation constant 12.0 instead of 11.3.

## THE MOLECULAR WEIGHT OF UREASE\*

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(Received for publication, May 31, 1938)

While urease was the first enzyme to be isolated, its molecular weight has not been determined thus far, although molecular weight determinations have been made with pepsin (1, 2) and the yellow respiratory enzyme (3) and recently with catalase (4-6). We considered it of interest to be able to compare the size of the urease molecule with that of other enzymes, as well as with other proteins in general. It also interested us to compare the molecular weights of the four crystallizable jack bean proteins, namely urease, canavalin, concanavalin A, and concanavalin B. Finally, it is hoped that at some later date knowledge of the molecular weights of enzymes may be of value when attempts are made to explain the mechanism of enzyme action.

### *Preparation of Crystalline Urease*

Jack beans rich in urease were purchased from C. O. Martin of Waldron, Arkansas, and were ground in the mill described in an earlier publication (7). Crystalline urease was prepared (8) and then recrystallized (9) from 32 per cent acetone. Later we observed that the recrystallization of urease could be carried out more rapidly and with more success from 30 per cent alcohol than from 32 per cent acetone.

Our first preparations of recrystallized urease were shown to be impure when ultracentrifuged, and we observed also that they became turbid upon standing in the ice-chest, and later gave heavy

\* Aided by grants from the Nobel, the Rockefeller, and the Wallenberg Foundations.

† John Simon Guggenheim Fellow, 1937-38.

precipitates of denatured protein. The urease crystals also became insoluble in water after being kept in the ice-chest. We then discovered that the phosphate buffer used for the recrystallization contained iron. The buffer was carefully purified by treatment with hydrogen sulfide as previously described (10). Urease recrystallized with purified buffer was of better quality but still had a tendency to become denatured upon being kept. The instability of purified urease solutions may have been caused by the presence of minute quantities of heavy metal acting as a catalyst for the oxidation of the —SH groups of the urease molecule. A second hypothesis is that the acetone employed may have contained traces of aldehyde. We used Kahlbaum's acetone and noted that this gave a very slight Schiff test for aldehyde. It was found at an earlier date that urease can be protected from inactivation by the addition of a mixture of sodium sulfite and sodium bisulfite. This would seem to support the theory that the denaturation of our urease solutions is brought about either by oxidation or by action of aldehydes.

Repeated recrystallizations of the urease with 32 per cent acetone were difficult because so much time was required and because so much of the urease became insoluble during the recrystallization. We always obtained successive crops of crystals when recrystallizing; the first crop was the best, while the later crops contained more insoluble material.

The purity of the crude and recrystallized urease was followed by determining the activity and by making micro-Kjeldahl determinations. We obtained values about the same as those originally reported; namely, about 100 urease units per mg. of crude urease and about 130 units per mg. of twice recrystallized urease.

#### *Determination of Sedimentation Constant*

The sedimentation constant for crystalline urease was determined in the ultracentrifuge as described by Svedberg (11). In all the experiments, the Lamm scale method (12) as modified by McFarlane (13) and by Pedersen (14) was used for following the rate of sedimentation.

In the first experiment urease was prepared from 900 gm. of jack bean meal. The crystals were washed with 30 cc. of ice-cold 32 per cent acetone containing phosphate buffer of pH 6.08,

and were then dissolved in 20 cc. of water. After centrifuging nearly clear, we added 12 cc. of M phosphate buffer at pH 6.08. The solution was chilled in the ice-chest, after which we added 8 cc. of acetone. The next day a good crop of urease crystals was found. The crystals were centrifuged off and stirred with 30 cc. of 0.9 per cent sodium chloride solution. They did not dissolve readily and appeared to be partly denatured.

A portion of the solution was centrifuged in the ultracentrifuge at 69,000 R.P.M. (centrifugal force about 350,000 times gravity). The results showed that the material consisted of five components with sedimentation constants of 7.4, 16.9, 19.0, 24.6, and  $27.0 \times 10^{-13}$  cm. per second dyne. The component with  $s_{20} = 19.0$  was present in the largest amount.

In the second experiment urease was prepared from 1500 gm. of meal. After the material was washed with cold 32 per cent acetone containing phosphate buffer of pH 6.08, the crystals were dissolved in 20 cc. of water and the solution was centrifuged in the cold room until nearly clear. We next added 12 cc. of M phosphate buffer of pH 6.08 and, after chilling, 9 cc. of acetone. The next day a good crop of crystals was obtained. These were centrifuged off and stirred with water. A considerable amount of the material did not dissolve and was observed to consist of denatured urease, since it was insoluble also in neutral phosphate solution.

An attempt was made to obtain crystals from the fraction of the crystals which had dissolved, but the precipitate obtained contained nothing but denatured protein.

In the third experiment urease was prepared from 1800 gm. of meal and was recrystallized from 32 per cent acetone by adding citrate buffer of pH 5.92 in 32 per cent acetone. The crystals were dissolved in 20 cc. of water and were recrystallized by chilling and adding 0.5 M citrate buffer but no acetone. The twice recrystallized urease was dissolved in 0.96 per cent phosphate buffer of pH 7.0 and centrifuged in the cold room until fairly clear.

An aliquot of this solution was run in the ultracentrifuge at 45,000 R.P.M. (centrifugal force about 150,000 times gravity). It showed a main component with  $s_{20} = 19.3$ . In addition there were two heavier components with  $s_{20} = 27.7$  and 36.0, but no lighter material. Another ultracentrifugation with the same solution gave similar results ( $s_{20} = 19.0$  for the main component).



The concentration of urease was 0.14 per cent and the activity 108 units per mg.

The mother liquor from which the urease for the third centrifugation was obtained gave a second crop of urease crystals when mixed with more acetone and allowed to stand in the ice-chest. These crystals were centrifuged off and dissolved in 10 cc. of neutral 0.96 per cent phosphate buffer. After this solution was centrifuged to free it from insoluble matter, it was run in the ultracentrifuge. This solution contained 0.71 per cent of urease and had an activity of 116 units per mg. It showed three components,  $s_{20} = 19.2, 27.1,$  and  $33.1$ . The component with  $s_{20} = 19.2$  was dominant in this experiment also, and no lighter material was revealed.

The urease for the fifth experiment was prepared from 3100 gm. of meal and was twice recrystallized from 32 per cent acetone with phosphate buffer of pH 6.07 which had been carefully freed from heavy metals by treatment with hydrogen sulfide. The crystals were dissolved in 0.96 per cent neutral phosphate to give a 1.12 per cent solution. The urease possessed 130 units per mg. An aliquot was ultracentrifuged at 45,000 R.P.M. (centrifugal force about 150,000 times gravity). The main component had  $s_{20} = 18.6$ . A heavier component with  $s_{20} = 26.3$  was still present, but only in a concentration of about 15 per cent of the total protein. Sedimentation curves for this experiment are given in Fig. 1.

The sixth experiment was carried out with a second crop of crystals which separated from the supernatant liquid from which the urease for the fifth experiment was obtained. This recrystallized urease was dissolved in 15 cc. of 0.96 per cent neutral phosphate buffer, and an aliquot was ultracentrifuged. The urease concentration was found to be 0.99 per cent and the activity 129 units per mg. The main component had  $s_{20} = 19.9$ . The heavier component had  $s_{20} = 26.1$ , and its concentration was about 25 per cent of the total protein. Consequently, the first crop of crystals gave a better result than the second crop.

The results of the six centrifugations show beyond doubt that urease is identical with the substance of sedimentation constant about 19, and that the urease employed in the fifth centrifugation was approximately pure.

*Determination of Diffusion Constant*

The same preparations of urease from which samples were taken for determination of the sedimentation constant were employed for measurement of the diffusion constant. Here the steel cell was employed according to the method of Lamm (12). Our urease preparations contained slight amounts of acetone or of alcohol

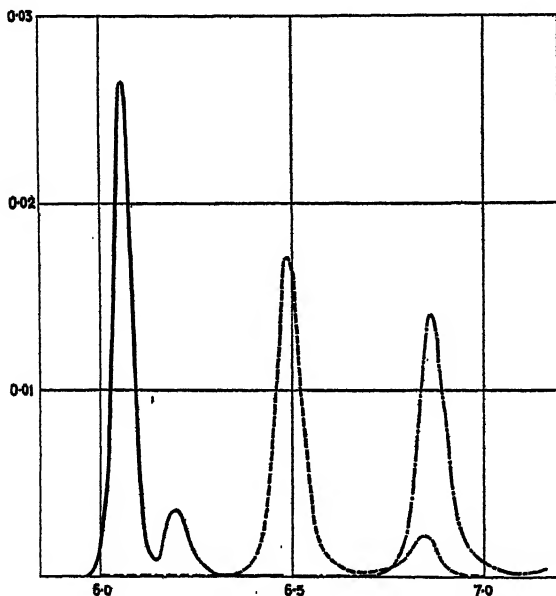


FIG. 1. Sedimentation curves of urease from the fifth experiment. Centrifugal force 150,000 times gravity. The three curves shown were obtained at 20, 50, and 73 minutes after reaching full speed. Sedimentation constants,  $18.6 \times 10^{-13}$  (for the main component) and  $26.3 \times 10^{-13}$ . The ordinate represents the scale line displacement measured in mm.; the abscissa, the distance from the center of rotation measured in cm.

and in order to obtain solutions suitable for diffusion measurements we placed 5 to 10 cc. in cellophane bags and dialyzed against 0.96 per cent neutral phosphate in the ice-chest for 12 to 24 hours before use. However, some urease solutions became opalescent upon being dialyzed overnight, while others became opalescent or even precipitated when kept 1 or 2 days in the apparatus for determination of the diffusion constant. The dialysis was not entirely

responsible for this denaturation, since urease kept in test-tubes also was denatured, although at a less rapid rate. The diffusion experiments were conducted at  $0^\circ$  in order to prevent denaturation of the urease, but since the urease diffused slowly our experiments required 3 to 4 days and the urease never remained unaltered over this period of time.

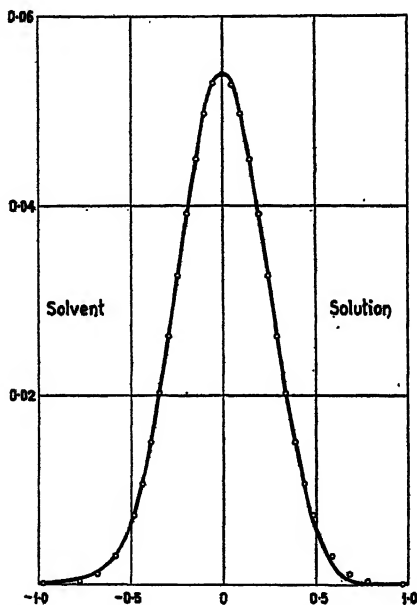


FIG. 2. Diffusion curve of urease. Time after starting, 43 hours 30 minutes. The experimental values are shown by the continuous curve; the points indicate the ideal diffusion curve of a homogeneous substance with  $D_{20} = 3.46 \times 10^{-7}$ . The ordinate represents the scale line displacement measured in mm.; the abscissa, the distance from the original boundary measured in cm.

We finally decided that it would not be possible to determine the diffusion constant for urease unless we could add some protective agent (15). Accordingly, the urease crystals were dissolved in a solution containing 0.96 per cent neutral phosphate, 0.78 per cent  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ , and 0.15 per cent  $\text{NaHSO}_3$ , and then dialyzed against this solution until the salt concentration was the same. This urease solution did not become opalescent after 2 weeks

dialysis. It was used successfully to determine the diffusion constant. The diffusion curve is shown in Fig. 2; the value found was  $D_{20} = 3.46 \times 10^{-7}$  sq. cm. per second. This value is corrected for the viscosity of the solvent and for the temperature in the ordinary way. That is

$$D_{20, \text{H}_2\text{O}} = D_{o.s} \times \frac{\eta_s}{\eta_{\text{H}_2\text{O}}} \times \left( \frac{\eta_{0^\circ}}{\eta_s} \right)_{\text{H}_2\text{O}} \times \frac{293.1}{273.1}$$

The viscosity of the solvent, referred to water, was measured in an Ostwald viscometer and was found to be  $(\eta_s/\eta_{\text{H}_2\text{O}})_{20^\circ} = 1.024$ .

#### *Determination of Partial Specific Volume*

After discovering that urease protected by sulfite could be dialyzed, we were able to use some of the dialyzed material to determine the partial specific volume. This was found to be 0.73.

#### *Calculation of Molecular Weight*

From the obtained values  $s_{20} = 18.6 \times 10^{-13}$ ,  $D_{20} = 3.46 \times 10^{-7}$ , and  $V = 0.73$ , we calculate a molecular weight of 483,000 for the urease with the equation (11)

$$M = \frac{RTs_{20}}{D_{20}(1 - V\rho)}$$

The sedimentation curves from the fifth experiment show that the preparation contained only a slight impurity. The diffusion curves are very close to the ideal distribution curves, which are obtained from the diffusion of a homogeneous substance. Therefore we conclude that the urease is a homogeneous protein.

#### SUMMARY

1. The preparation and recrystallization of urease from jack bean meal are described.
2. The sedimentation constant was determined. Several components were present, but the main component always had a sedimentation constant of about 19. The preparation which possessed the highest activity, 130 units per mg., contained the least impurity and gave the value  $s_{20} = 18.6 \times 10^{-13}$  cm. per second dyne.
3. By employing urease solution protected with sulfite, we

were able to determine the diffusion constant. This was  $D_{20} = 3.46 \times 10^{-7}$  sq. cm. per second.

4. The partial specific volume was found to be 0.73.

5. The molecular weight of urease was calculated to be 483,000. The experiments show that the urease is a homogeneous protein.

The authors wish to express their thanks to Professor The Svedberg for his kind interest during the work.

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## THE MOLECULAR WEIGHTS OF CANAVALIN, CON-CANAVALIN A, AND CONCANAVALIN B

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Having determined the molecular weight for crystalline urease (1), we wished to determine the molecular weights of the three other crystallizable proteins (2) of the jack bean, namely canavalin, concanavalin A, and concanavalin B, and to compare the four values. We have been especially interested to learn the molecular weight for concanavalin A because this globulin is a hemagglutinin; indeed, it is the only hemagglutinin obtained thus far in pure and in crystalline condition (3, 4). Concanavalin A has been found to contain 0.023 per cent of manganese after two recrystallizations and it was of interest to see whether the manganese content bore any stoichiometrical relationship to the molecular weight. Preparation and recrystallization of canavalin (5), concanavalin A (3), and concanavalin B (5) were carried out as previously described, with jack bean meal residues left after extraction of the urease. It should be noted that we have employed the term "canavalin" for the crystallizable protein obtained after incubating crude canavalin with trypsin.

Centrifugation and diffusion experiments showed that these three globulins are homogeneous with respect to their molecular weights. The centrifugations were carried out in a 12 mm. cell, while the diffusion experiments were all made in a 10 mm. cell at 20°.

*Canavalin*.—A 1.37 per cent solution of once recrystallized canavalin in 5 per cent sodium chloride was centrifuged at 68,000 R.P.M. (centrifugal force about 340,000 times gravity). The sedimentation curves are shown in Fig. 1. The sedimentation constant was  $s_{20} = 6.4 \times 10^{-13}$  cm. per second dyne. A diffusion experiment with a 0.68 per cent solution of once recrystallized canavalin in

5 per cent sodium chloride gave  $D_{20} = 5.1 \times 10^{-7}$  sq. cm. per second. The diffusion curve is shown in Fig. 2.

The partial specific volume of a 0.75 per cent solution of the canavalin in 5 per cent sodium chloride was found to be 0.73.

The molecular weight of canavalin is calculated to be 113,000.

*Concanavalin A*—A twice recrystallized solution of concanavalin A was diluted until the salt concentration was 5 per cent and

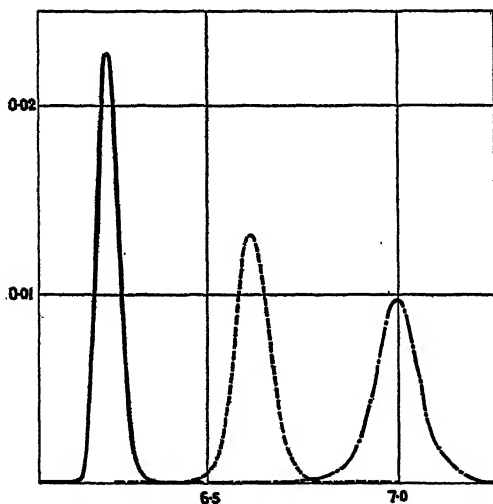


FIG. 1. Sedimentation curves of canavalin. Centrifugal force 340,000 times gravity. The three curves shown were obtained at 17, 47, and 76 minutes after reaching full speed. Sedimentation constant  $6.4 \times 10^{-13}$ . The ordinate represents the scale line displacement measured in mm.; the abscissa, the distance from the center of rotation measured in cm.

the protein concentration was 1.45 per cent. Before we could centrifuge, some of the concanavalin A had crystallized out. The solution was centrifuged at 60,000 R.P.M. (centrifugal force about 260,000 times gravity). The sedimentation constant was  $s_{20} = 6.0 \times 10^{-13}$  cm. per second dyne.

For determination of the rate of diffusion, the concanavalin A was diluted to contain 0.7 per cent protein in 5 per cent NaCl, 0.17 per cent  $\text{Na}_2\text{PO}_4$ , and 0.07 per cent  $\text{KH}_2\text{PO}_4$ . The diffusion constant was  $D_{20} = 5.6 \times 10^{-7}$  sq. cm. per second. The

specific volume was found to be 0.73, with an 11 per cent solution of concanavalin A in saturated sodium chloride. The molecular weight is calculated to be 96,000. This value bears no simple relationship to the content of manganese.

*Concanavalin B*—A twice recrystallized solution of 1.2 per cent concanavalin B in 5 per cent NaCl, 0.46 per cent  $\text{Na}_2\text{HPO}_4$ , and

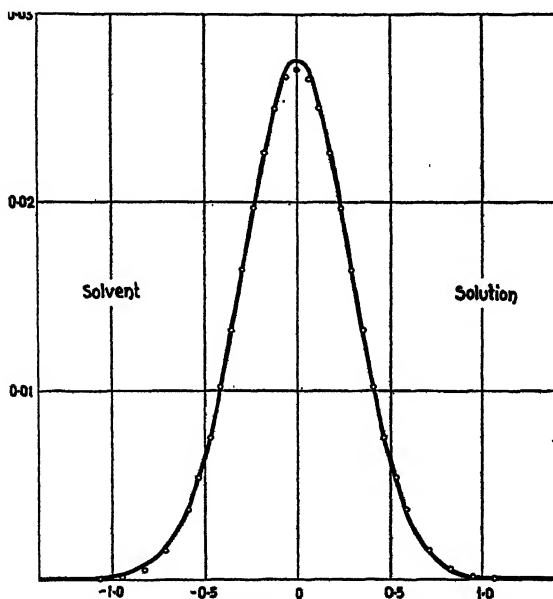


FIG. 2. Diffusion curve of canavalin. Time after starting, 24 hours. The experimental values are shown by the continuous curve; the points indicate the ideal diffusion curve of a homogeneous substance with  $D_{20} = 5.1 \times 10^{-7}$ . The ordinate represents the scale line displacement measured in mm.; the abscissa, the distance from the original boundary measured in cm.

0.18 per cent  $\text{KH}_2\text{PO}_4$  was centrifuged at 69,000 R.P.M. (centrifugal force about 350,000 times gravity). The sedimentation constant was found to be  $s_{20} = 3.49 \times 10^{-13}$  cm. per second dyne.

The solution employed for determining the rate of diffusion contained 0.4 per cent concanavalin B, 5 per cent NaCl, 0.46 per cent  $\text{Na}_2\text{HPO}_4$ , and 0.18 per cent  $\text{KH}_2\text{PO}_4$ . The diffusion constant found was  $D_{20} = 7.4 \times 10^{-7}$  sq. cm. per second.



The partial specific volume of concanavalin B was found to be 0.73, and the molecular weight is calculated to be 42,000.

#### SUMMARY

Three of the jack bean globulins, canavalin, concanavalin A, and concanavalin B, have been ultracentrifuged and shown to be pure substances. Their molecular weights respectively are 113,000, 96,000, and 42,000.

We thank Professor The Svedberg for the privilege of working in his laboratory. We desire to acknowledge our indebtedness to the Guggenheim Foundation and to the Wallenberg Foundation for financial assistance.

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## THE UTILIZATION OF PYRUVIC ACID BY THE DOG

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According to the Embden-Meyerhof hypothesis, pyruvic acid is the immediate precursor of lactic acid in muscle glycolysis. Krebs (13) showed that various tissues under anaerobic conditions will convert 40 per cent of added pyruvic acid to lactic acid, although, under aerobic conditions, the products are carbon dioxide and water. Jost (12) and Lawson (14) found that the cortex of the kidney can readily reduce pyruvic acid to lactic acid. In the non-phosphorylating glycolysis of the young embryo, however, pyruvic acid is formed but is not changed to lactic acid (Needham and coworkers (19, 20)). Elliott (6, 7) and others found that the renal cortex, and Peters (21) that brain tissue, can oxidize lactic acid to pyruvic acid.

Studies of the rôle of pyruvic acid in carbohydrate metabolism *in vivo*, with the exception of cases of vitamin B<sub>1</sub> deficiency, are relatively few. It has been shown that pyruvic acid can be converted to glucose in fasting rabbits by Mayer (18) and in phlorhizinized dogs by Ringer (23) and by Dakin and Janney (5). Pyruvic acid, however, is not a source of blood sugar in the hepatectomized dog (16). It may be reduced to lactic acid, as shown by Mayer (18) in 1912, and again in 1937 by Margaria and Ponzio (17) and by Simola (26).

In the present investigation a study was made of the rate at which pyruvic acid can be utilized by the dog when given by continuous intravenous injection. The possible effects on the content of lactic acid, glucose, and inorganic phosphate of the blood and urine were also studied. Normal dogs, and others in which some abnormality of carbohydrate metabolism might be present, owing to prolonged fasting, pancreatectomy, or adrenalectomy, were used in these studies. It was considered of interest also to know whether the accumulation of lactic acid in the blood

produced by other means than severe exercise (11), such as by injections of glucose, levulose, or epinephrine, might also be accompanied by an increase in the concentration of pyruvic acid.

#### EXPERIMENTAL

Pyruvic acid, freshly redistilled *in vacuo*, was neutralized with sodium hydroxide and injected intravenously (as a 5 or 10 per cent solution) into dogs at the rate of 0.25, 0.5, or 1.0 gm. per kilo of body weight per hour. Pyruvic acid was administered to normal animals 16 to 20 hours after food had been taken, and to dogs which had been fasted for from 12 to 14 days in order to develop hunger diabetes. Adrenalectomized dogs had been maintained with cortin for from 6 months to 3 years and were deprived of treatment 1 to 3 days prior to each experiment. Depancreatized dogs were maintained with insulin for 1 or 2 months and deprived of insulin for 2 and 3 days before each experiment.

Additional studies included similar injections of sodium lactate, sodium bicarbonate, sodium chloride, glucose, levulose, and epinephrine. Specimens of blood and urine obtained at specified intervals were used for the following determinations: pyruvic acid, by the Case (3) and by the Peters and Thompson (22) modifications of the Simon and Neuberg (27) procedure; lactic acid, by the method of Friedemann, Cotonio, and Shaffer (9); glucose, by a modified method of Folin (8) on unlaked blood; and levulose, by the method of Roe (24).

#### Results

Injections of pyruvic acid increased the pyruvic acid content of the plasma to from 6 to 20 mg. per 100 cc. when 0.25 or 0.5 gm. per kilo per hour was used, and to as high as 76 mg. when 1.0 gm. was given. Normal values were again obtained 3 or 4 hours after the injection was discontinued (Table I). A smaller increase occurred in the cells (Table II). The urinary excretion of pyruvic acid of approximately 0.4 mg. per hour increased rapidly during injection and returned to normal amounts at about the same time after injection as the excess disappeared from the blood. The pyruvic acid recovered in the urine amounted to from 4.2 to 22.8 per cent of that injected. Other substances, such as  $\alpha$ -ketoglutaric acid (26), may be included in the pyruvic acid

TABLE I

*Concentration in Blood and Urinary Excretion of Pyruvic and Lactic Acids Resulting from Injection of Sodium Pyruvate*

Dog No.	Condition	Pyruvic acid injected	Time after injection							Urinary excretion* Pyruvic acid, per cent
			0	15 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	6 hrs.	
			Pyruvic acid in blood, mg. per 100 cc.							
		gm. per kg. per hr.								
1	Normal	0.25	1.3	5.1	3.9	6.7	6.2†	1.7	2.7	4.2
2	"	0.5	1.8		20	19†		2.0	2.1	10.4
3	"	1	1.4	19	72		73†		10	22.8
			Lactic acid in blood, mg. per 100 cc.							Lactic acid, per cent
1	"	0.25	21	24	26	34	43†	33	21	1.9
2	"	0.5	18		17	30†		18	9.7	2.0
3	"	1	18	40	45		65†		16	4.4
			Pyruvic acid in blood, mg. per 100 cc.							Pyruvic acid, per cent
4	Fasted 12 days	0.5	1.4	8.9	19	27†		3.8	2.0	10.1
5	" 14 "	1	0.9	24	56	86		22	4.2	12.4
6	Depancreatized	0.5‡	0.9	4.8	38	32		37	40	10.4
7	"	0.5§	1.4		16	28		3.9	1.7	9.5
8	"	1‡	1.8	14	34	52		44	38	13.6
9	Adrenalectomized	0.5	1.4		9.4	31		6.8	2.6	12.4
10	"	0.5¶	1.3		30	43		12	2.7	15.5
11	"	0.5¶	1.3		31	38		7	3.7	18.3
			Lactic acid in blood, mg. per 100 cc.							Lactic acid, per cent
4	Fasted	0.5	16	22	39	38		20	10	0.9
5	"	1	17	21	63	85		53	20	6.2
6	Depancreatized	0.5	57	68	90	128		78	53	4.8
7	"	0.5	39		62	91		38	33	17.0
8	"	1	31	36	43	48		104	96	7.8
9	Adrenalectomized	0.5	12		15	17		10	5	1.0
10	"	0.5	14		22	29		16	14	0.3
11	"	0.5¶	10		22	29		9	15	1.0

\* Excretion during the 6 hours of the experiment calculated as equivalent percentage of pyruvic acid injected.

† End of injection.

‡ Off insulin 3 days.

§ Off insulin 2 days.

|| Off cortin 3 days.

¶ Off cortin 1 day.

determinations of blood and urine, but it seems reasonable to suppose that the values obtained following injection of pyruvic acid represent chiefly pyruvic acid.

There was a concomitant rise in the lactic acid content of the blood and urine during the injection of pyruvic acid, and from 0.7 to 9.5 per cent of the injected pyruvic acid could be accounted for as lactic acid in the urine. When sodium pyruvate was incubated with blood *in vitro*, no reduction to lactic acid occurred.

When pyruvic acid was injected into fasting, depancreatized, or adrenalectomized dogs, in all cases the concentration in the blood at the end of the injection was about twice that found in normal dogs, but normal values were obtained at similar times after discontinuation of the injection except in cases complicated by severe

TABLE II  
*Distribution of Pyruvic Acid in Blood*

Time after injection	Hematocrit	Plasma	Whole blood	Erythrocytes
<i>hrs.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
0	57.8	1.9	1.2	0.7
1	51.0	13.8	8.7	3.8
2*	48.2	15.4	10.1	4.4
4	53.4	2.8	2.3	1.9

\* End of injection of 0.5 gm. of Na pyruvate per kilo per hour.

alkalosis. The urinary excretion of pyruvate was somewhat higher than in normal dogs, but approximately 80 to 90 per cent was retained. Reduction of pyruvic acid to lactic acid occurred in all of these dogs, although to a smaller extent in the adrenalectomized animals.

The injection of lactic acid increased the lactic acid content of the blood to a greater extent than pyruvate injections increased the blood pyruvate, but similar amounts were recovered in the urine (Table III). The pyruvate content of the blood was unaltered by injections of lactic acid, but minor increases in the pyruvate occurred in the urine. Neither pyruvate nor lactate injections altered the glucose content of the blood, but both produced a decrease in the inorganic phosphate of the blood. In contrast to the administration of glucose, however, pyruvate

TABLE III

*Concentration in Blood and Urinary Excretion of Pyruvic and Lactic Acids Resulting from Injection of Sodium Lactate*

Dog No.	Condition	Pyruvic acid injected	Time after injection							Urinary excretion* Pyruvic acid, per cent
			0	15 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	6 hrs.	
			Pyruvic acid in blood, mg. per 100 cc.							
		gm. per kg. per hr.								
13	Normal	1	1.0		2.7	2.8†		1.5	1.2	0.5
14	"	1	1.2		2.7	2.7†		1.8	1.8	0.2
			Lactic acid in blood, mg. per 100 cc.							Lactic acid, per cent
12	"	0.25	14	28	45	61	55†	29	9.2	10.2
13	"	1	24	47	109	134†		46	24	18.0
14	"	1	15	62	105	167†		57	30	19.0
			Pyruvic acid in blood, mg. per 100 cc.							Pyruvic acid, per cent
6	Depancreatized	0.5‡	1.2		2.5	2.6†		1.9		0.3
9	Adrenalectomized	0.5§	1.0		1.7	1.7		1.0	1.1	0.2
10	"	0.5	1.1		1.2	1.9		1.1	1.2	0.1
11	"	0.5¶	1.2		1.0	1.1		1.3	1.1	0.4
			Lactic acid in blood, mg. per 100 cc.							Lactic acid, per cent
6	Depancreatized	0.5	38		96	112		18		23.5
9	Adrenalectomized	0.5	27		114	87		27	24	16.4
10	"	0.5	7.6		63	79		34	15	10.9
11	"	0.5	13		67	99		36	23	10.5

\* Excretion during the 6 hours of the experiment calculated as equivalent percentage of lactic acid injected.

† End of injection.

‡ Off insulin 3 days.

§ Off cortin 3 days.

|| Off cortin 1 day.

¶ Off cortin 2 days.

produced but a very slight decrease in the phosphate excretion, while the effect of lactic acid appeared more comparable to that of glucose.

Certain substances known to increase blood lactic acid *in vivo*, such as glucose, levulose (Wierzuchowski and Laniewski (28)), and epinephrine (4), were also injected into normal dogs. With each of these the pyruvate of the blood was approximately doubled. Injections of physiologic saline or sodium bicarbonate solution did not alter the pyruvate content of the blood.

### *Comment*

The utilization of ingested carbohydrate is generally determined from a study of the concentration in the blood and the amount excreted in the urine. On this basis it appears that pyruvic acid is well utilized by the dog. During the 6 hour experimental period, which included the time of injection of pyruvic acid and the period of recovery, the concentration in the blood returns practically to normal, as does the rate of excretion of this substance in the urine. From the amount excreted, either as pyruvic or lactic acid, during this period it is evident that the normal dog can retain 88 to 96 per cent of the pyruvic acid injected when it is given at the rate of 0.5 gm. per hour, or at least 73 per cent when it is given at the rate of 1 gm. per hour. Whether the amount retained was utilized in oxidation or storage in some other form was not determined. Even in the dogs that had been fasted, depancreatized, or adrenalectomized, the average retention was 82 per cent. The relative amounts excreted in the urine as pyruvic or lactic acid were extremely variable and either acid might be the predominant one.

The observation that during the utilization of pyruvic acid lactic acid is markedly increased in both blood and urine is in agreement with earlier work. The increases in blood lactic acid are rapid and may reach levels as high or even higher than that of the pyruvic acid that is being injected. Pyruvic acid may thus be grouped with the other carbohydrates, glucose, levulose, and dihydroxyacetone, which on administration produce increases in blood lactic acid (10).

The reduction of pyruvic acid to lactic acid did not occur in blood *in vitro*. Sherman and Elvehjem (25) have also shown that pyruvic acid is not metabolized by blood *in vitro*, but Wilkins, Weiss, and Taylor (29) found that pyruvic acid disappeared upon incubation with blood.

Studies on adrenalectomized dogs show that they can utilize about the same percentage of injected pyruvate or lactate as the normal dog. They can reduce pyruvic to lactic acid, although the amount excreted as lactate is never large. Earlier work by Buell, Anderson, and Strauss (2) showed that the adrenalectomized rat did not utilize sodium lactate when given orally as well as did the normal rat. The depancreatized dog utilizes most of the injected pyruvate. The marked increase in lactate produced thereby is of interest in contrast to the absence of lactate formation following injections of glucose into such dogs. Fasting for 2 weeks did not make any appreciable change in the ability of the dog to utilize pyruvic acid, although Lipschitz, Potter, and Elvehjem (15) found that in fasted birds there is a lowered rate of pyruvate removal from the blood. The reverse process, the oxidation of lactic acid to pyruvic acid, does not occur to any appreciable extent in the intact dog.

The absence of any increase in blood sugar during the injection of either pyruvate or lactate is similar to the observation made by Abramson, Eggleton, and Eggleton (1) on lactate. The small increase in pyruvic acid which accompanies a much larger increase in lactic acid when glucose, levulose, or epinephrine is administered is of interest. It is similar to the increase in pyruvic acid in blood found after severe exercise by Johnson and Edwards (11). Just why a similar increase in pyruvic acid does not occur when lactic acid is produced by blood glycolysis, or along with the high level of lactic acid in the depancreatized dog, is not evident.

#### SUMMARY

Pyruvic acid is rapidly utilized by the dog. During this process there is an increase in lactic acid in the blood and urine. This increase also occurs after a prolonged fast, pancreatectomy, or adrenalectomy. The reduction of pyruvic acid to lactic acid does not occur in blood *in vitro*.

When lactic acid was injected, there was no evidence in the blood of oxidation of the lactic acid to pyruvic acid although there was a small increase in urinary pyruvic acid.

Substances which produce a small increase in pyruvic acid along with a larger increase in blood lactic acid are glucose, levulose, and epinephrine.



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# THE EFFECT OF THE INTRAVENOUS INJECTION OF GLUCOSE AND OTHER SUBSTANCES ON THE CONCENTRATION OF POTASSIUM IN THE SERUM OF THE DOG

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Coincident with the drop in blood sugar produced by insulin, there occurs a marked drop in the inorganic phosphate and potassium in the blood serum and urine. This was shown by Harrop and Benedict in 1923 (1, 2) and by Briggs, Koechig, Doisy, and Weber (3). Kerr (4) showed also that the potassium that disappeared did not enter the corpuscles. Because an increased intake of carbohydrate will produce a decrease in inorganic phosphate in the serum, it might be expected that it would also produce a decrease in the concentration of potassium. To test this the effect of the administration of glucose and levulose to dogs has been studied; the influence of lactic acid and pyruvic acid, of epinephrine, and of fasting and pancreatectomy has also been investigated.

## EXPERIMENTAL

Dogs, fasted usually from 16 to 20 hours, were given injections of these various substances at a constant rate with the Woodyatt pump for periods of 2 or 3 hours. Samples of blood and urine were collected at intervals during injection and during the 3 or 4 hour recovery period. The preliminary urine was an overnight specimen. Duplicate analyses were made for the substance given, and for sodium, potassium, inorganic phosphate, and chloride in the blood and urine.

Potassium was determined by the method of Shohl and Bennett (5) as modified by Hald (6) and Hartzler (7); sodium was deter-

mined directly in the urine and on ashed serum by the method of Barber and Kolthoff (8) as modified by Butler and Tuthill (9). Inorganic phosphate was determined by the methods of Fiske and Subbarow (10) for urine and of Kuttner and Lichtenstein (11) for blood. The modified Volhard-Harvey titration (12) was used for chlorides in the urine and the microadaptation of this by Keys (13) for blood chlorides. The method of Folin (14) was used for blood sugars.

### *Results*

Six normal dogs were given continuous intravenous injections of glucose for 3 hours, one at the rate of 0.25 gm., four at the rate of 1 gm., and one at the rate of 2 gm., per kilo of body weight per hour. All six dogs showed a marked decrease in the potassium and inorganic phosphate content of the serum. The decrease in potassium varied from 13 to 25 per cent, with an average of 20 per cent; the decrease in phosphate varied from 23 to 64 per cent, with an average of 46 per cent. The sodium always showed a greater decrease in milliequivalents per liter than did the potassium, but when the decrease was figured as percentage of the original value, it was very small. It was never greater than the percentage drop in the hematocrit value and usually was much smaller. Changes in blood chlorides were even smaller and were of no significance (Table I).

When samples of blood were taken at the same intervals but from dogs that had not been given an injection of glucose, an average value of 4.5 milliequivalents of potassium was found. There was no decrease during an interval of 6 hours even though a decrease in the hematocrit values might occur after the first venipuncture which was about equal to the decrease observed in the injection experiments.

Levulose was administered to three normal dogs, to one at the rate of 0.25 gm., and to two at the rate of 1 gm., per kilo per hour. Again marked decreases in potassium and phosphate occurred without significant changes in sodium or chloride. The decrease in potassium varied from 14 to 20 per cent, with an average of 18 per cent.

Epinephrine in physiologic saline solution was administered to three normal dogs at the rate of 0.01, 0.1, and 0.2 mg. per hour,

TABLE I

*Effect of Injections of Glucose on Electrolytes in Serum of Normal Dog*

Dog No.	Glucose	Time	Blood sugar	Potassium	Decrease	Sodium	Decrease	Phosphate	Decrease	Chlorides	Decrease	Hematocrit	Decrease
	gm. per kg. per hr.	hrs.	mg. per 100 cc.	m.eq.	per cent	m.eq.	per cent	m.eq.	per cent	m.eq.	per cent	per cent	per cent
1	0.25	0	64	5.0		148		1.3		111			
		$\frac{1}{2}$	74	4.0		148		1.3		112			
		1	64	4.6		143		1.2		111			
		2	71	4.1		143		1.1		110			
		3*	71	3.8	24	144	2.7	1.0	23	110	0.9		
		4	65	4.4				1.4		110			
		6	63	4.9				1.4		112			
		0	61	4.5		148		1.4		106			
		$\frac{1}{2}$	150	3.9		148		1.1		107			
2	1	1	82	2.9		134		0.7					
		3*	80	3.4	25	144	2.7	0.5	64	107	+0.9		
		6	67	4.6		148		1.5		106			
		0	81	4.3		147		1.4		108			
		$\frac{1}{2}$	156	3.4		144		1.1		105			
3	1	1	198	3.5		144		0.7		105			
		3*	168	3.4	21	143	2.7	0.5	64	104	3.7		
		6	66	4.8		144		1.8		105			
		0	66	5.0		146		1.0		111		45.0	
		$\frac{1}{2}$	148	4.5		143		0.6		109			
4	1	3*	102	4.2	16	142	2.7	0.6	40	110	0.9	42.3	6.0
		4	65	4.7		145		0.8		109		42.5	
		6	69	5.3		145		1.4		109		41.2	
		0	57	5.0		148		1.0		108		50.3	
		$\frac{1}{2}$	148	4.7		140		0.9		107		45.4	
5	2	1	102	4.1		142		0.5		107		44.2	
		2	145	3.7		138		0.5		105		46.2	
		3*	146	3.8	24	134	9.5	0.4	60	104	3.7	45.4	9.7
		4	53	4.8		141				109		47.4	
		6	72	5.0		143				108		44.5	
6	1†	0	67	4.5		148		0.8		111		46.2	
		1	104	3.8		144		0.6		110		45.2	
		3*	95	3.9	13	142	4.0	0.6	25	108	2.7	46.2	0
		4	62	4.6		147		1.2		111		44.4	
		6	67	4.7		146		1.5		110		44.0	

\* Injection discontinued.

† With cortin.

respectively, by continuous injection for 2 and 3 hours. This quantity of epinephrine was as effective as the glucose and levulose in decreasing the concentration of potassium and phosphate in the serum; it also produced no effect on the sodium or chloride content. The decreases in potassium varied from 19 to 32 per cent.

When lactic or pyruvic acid, usually as the sodium salt, was administered by continuous injection for 2 or 3 hours to thirteen normal dogs, marked decreases in the level of potassium and phosphate in the serum were found. The sodium or ammonium ion administered with these acids, however, introduced a complicating factor in the interpretation of the results. The decreases in potassium varied from 11 to 34 per cent, with an average of 18 per cent. These changes were associated with increases in the concentration of sodium in the serum of from 8 to 32 per cent when the sodium salts were used, although the concentration of sodium was unchanged when the ammonium salts were injected.

Sodium bicarbonate was given to three dogs at the rate of 0.2 and 0.25 gm. per kilo per hour for 3 hours. The amount of sodium injected was approximately equal to that injected with the lactic or pyruvic acid when they were given at the rate of 0.25 gm. per kilo per hour. These experiments showed that the injection of sodium bicarbonate decreased the concentration of potassium and phosphate just as effectively as did the sodium salts of the organic acids, even though the increases in serum sodium, which had a range of from 2 to 6 per cent, were much smaller. A further attempt to determine the effect of sodium without an increased alkalinity involved the administration of sodium chloride. The salt was given to four dogs in a 1.75 per cent solution at the rate of 0.3 gm. per kilo for 3 hours. The amount of sodium given was roughly twice that given as bicarbonate. While in two cases this did not produce a significant change in potassium, in two cases there were decreases of 17 and 21 per cent.

An attempt was made to develop "hunger diabetes" in two dogs by fasting them 11 and 14 days. Glucose was then administered at the rate of 1 gm. per kilo per hour for 3 hours. A blood sugar curve typical of diabetes was obtained for one dog: there was an 11 per cent decrease in the concentration of potassium in the serum. In the other dog the blood sugar increased less, while the level of potassium decreased 17 per cent. Glucose was also

administered at the same rate to four depancreatized dogs which were maintained without insulin for 3 days. The usual response of phosphate to the injection of glucose was abolished or occurred to only a slight extent in the cases of these four dogs and the response of potassium was markedly diminished in two dogs. The other two dogs, which showed a normal response of potassium to

TABLE II

*Urinary Electrolytes before and after Injection of Glucose into Normal Dogs*

Dog No.	Weight	Glucose	Time	Urine volume	Average hourly output			
					K	Na	P	Cl
	kg.	gm. per kg. per hr.	hrs.	cc.	mg.	mg.	mg.	mg.
1	11.8	0.25	16.6*	495	30	126	21	168
			3†	36	35	26	4	
			3‡	278	31	70	16	64
2	13.3	1	16.8	215	34	98	18	116
			3	159	12	14	1	24
			3	275	97	145	11	162
3	11.9	1	16.4	128	31	62	14	79
			3	22	33	16	1	40
			3	320	86	182	8	145
4	12.9	1	16.7	150	35	80	15	97
			3	210	24	12	2	32
			3	176	76	110	6	109
5	11.2	2	17.2	132	27	64	12	91
			3	210	41	29	0.4	44
			3	136	54	41	9	31
6	11.4	1§	15.6	125	26	56	17	94
			3	95	27	24	2	7
			3	170	38	24	11	38

\* Control urine, overnight specimen.

† Urine collected during injection.

‡ Urine collected following injection.

§ Large quantities of cortin also given.

glucose, were in a severe condition and died within a few hours of the end of the injection in spite of insulin treatment.

Although the potassium and inorganic phosphate of the serum in normal dogs decrease simultaneously on the injection of glucose, no such similarity exists in the changes in the urinary output of these substances. On the contrary, the output of phosphate in

the urine falls off very rapidly and usually disappears entirely, whereas the output of potassium is not consistent. The excretion of potassium may continue during the injection at the same average hourly rate as during the overnight control period, or it may be higher or lower. Moreover, the decrease in the excretion of potassium is never as great as that which occurs in the excretion of sodium (Table II).

### *Comment*

The administration of glucose produces a decrease in the concentration of potassium in the serum of the dog just as definitely as a decrease in inorganic phosphate. The effect of increased utilization of carbohydrate on these electrolytes is similar whether produced by an increased intake of carbohydrate or by insulin. Levulose, which is very readily used by the animal organism, produces the same response as glucose.

Earlier work has shown that epinephrine has an effect on the concentration of phosphate which is similar to that of glucose or insulin (15). Results reported concerning its effect on serum potassium are variable. D'Silva (16), Schwarz (17), Marenzi and Gerschman (18), and others have reported a rapid but transient increase in the potassium content of the serum of cats, rats, and dogs on the injection of epinephrine with, in some cases, a subsequent decrease below normal. Keys (19, 20) substantiated the findings of the transient increase in rabbits, cats, and dogs but, in human beings, found an immediate and marked fall in the concentration of potassium. In our investigation of the effect of long continued injection of epinephrine the same decrease in potassium concentration was found as from insulin or glucose. The decrease in the concentration of potassium which parallels the well known decrease in the concentration of phosphate is undoubtedly of more importance in relation to the effect of epinephrine on carbohydrate metabolism than is the transient rise that may be elicited in certain animals.

It is very difficult to determine the effect of pyruvic and lactic acids, as they cannot be given in sufficient quantity as such; when they are given in the form of the sodium salts, as in these experiments, ion antagonism must be considered. Although a marked decrease in potassium was produced, this was no greater

when 1 gm. of the acids, as the sodium salts, was given per kilo per hour than when 0.25 gm. was given or when sodium bicarbonate was given. The changes found appear to represent the maximal effect which can be produced by the injection of sodium ions. It is interesting to note that the effect of carbohydrate alone on potassium concentration may be just as great as this.

Pancreatectomy, which diminishes or abolishes the response of phosphate to glucose, does not always have such a clear cut effect on the response of potassium. In fact, in the cases of the two dogs in severe diabetes the phosphate of the serum was unchanged by glucose whereas the potassium concentration showed a decrease comparable to that produced in normal dogs by glucose.

Increased utilization of carbohydrate affects the concentration of potassium in the serum of the normal dog without showing any definite influence on the urinary excretion. In contrast to this the concentration of inorganic phosphate in both blood serum and urine is markedly decreased. It thus appears that carbohydrate either has a less direct effect on potassium than phosphate or that other factors, which control the concentration of potassium, tend to mask the effect of carbohydrate.

#### SUMMARY

The administration of glucose produces a decrease in the concentration of potassium in the serum, although this decrease, expressed as a percentage of the original, is not as large as that produced simultaneously in inorganic phosphate. The administration of levulose or epinephrine produces the same response.

Sodium lactate or sodium pyruvate produces a similar decrease, but this can be accounted for as an effect of the sodium, since sodium bicarbonate is equally effective. Decreases may be produced in some dogs even by sodium chloride.

In the case of depancreatized dogs without insulin the injection of glucose may or may not change the concentration of potassium in the serum.

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## THE ISOLATION OF NUCLEIC ACID AND NUCLEOPROTEIN FRACTIONS FROM PNEUMOCOCCI

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Several attempts have been made in the past to isolate the nucleic acids and nucleoproteins of various bacterial species (Galeotti (1), Ruppel (2), Levene (3), Johnson and Brown (4), Schaffer, Folkoff, and Bayne-Jones (5), Stull (6), Coghill (7), Ferramola (8)). In general the methods used have involved the extraction at alkaline reaction of whole cells, or of cells disintegrated by mechanical grinding or by autolysis, followed by precipitation of the alkaline extract with acid. As Heidelberger and Kendall (9) have pointed out, however, prolonged exposure to an alkaline reaction may profoundly modify nucleoproteins and in particular cause the loss of nucleic acid. To minimize these alterations, these authors extracted hemolytic streptococci, previously disintegrated by grinding in the cold, with a buffer mixture at pH 6.5 and thus separated a nucleoprotein fraction characterized by a high phosphorus content (3 to 4 per cent). In the present study, both a ribonucleic acid and a ribonucleoprotein fraction have been released from pneumococci at neutral reaction by an enzymatic process which does not involve disintegration of the cell structure.

### EXPERIMENTAL

*Bacterial Cells*—All the experiments reported in this paper were performed with an R culture derived from Type II pneumococci. The cells were grown for 5 hours in a meat infusion-peptone broth,

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and separated from the medium in a steam-driven, sterilizable Sharples centrifuge. Details of the bacteriological technique are described elsewhere (10).

*Analytical Methods*—Nitrogen has been estimated by the method of Conway and Byrne (11); the sulfuric acid and sodium hydroxide used in the titrations were standardized at frequent intervals throughout the course of the work. Phosphorus has been estimated by a modification of the colorimetric method of Youngburg and Youngburg (12). Digestion with sulfuric acid and superoxol was carried out for 40 minutes both for nitrogen and for phosphorus determinations. All estimations were made in duplicate. The presence of pentoses was tested for with Bial's orcinol reagent. The Feulgen reaction (13) was carried out according to the technique described by Widström (14).

*Method Used for Separation of Nucleic Acid and Nucleoprotein from Pneumococci*—It has been shown elsewhere (10), that when pneumococci are maintained in acetic acid at pH 4.2 for 24 hours and then resuspended at neutral reaction, they undergo a slow change from the Gram-positive to the Gram-negative state; this change in staining reaction is not accompanied by an actual disintegration of the bacterial cells, although they become smaller in size. It is from the material released into solution during the course of this change that a nucleic acid and a nucleoprotein fraction have been separated.

As a matter of practise the cells from 100 liters of pneumococcus culture were resuspended in 200 cc. of 0.1 M acetate buffer at pH 4.2 and allowed to stand in this medium at 37° overnight. The cells were next centrifuged and resuspended in 400 cc. of 0.4 per cent sodium chloride solution; the reaction was adjusted to pH 7.0 by the cautious addition of sodium hydroxide; care was taken not to allow the cell suspension to become alkaline at any time in the course of neutralization. The neutral suspension was then incubated at 37° until the cells had become Gram-negative. This state was often reached within 4 to 7 hours; in a few experiments, however, a more prolonged incubation was required. At the end of the incubation period the cells were separated from the neutral medium by centrifugation and the supernatant was filtered through a Berkefeld filter (V). The filtrate was adjusted to pH 4.2 by the addition of 50 per cent acetic acid. A precipitate formed

(Fraction A) and was removed by centrifugation. The acid supernatant at pH 4.2 was then brought to approximately pH 2.5 with 1 N HCl; a new precipitate formed and was also separated by centrifugation (Fraction B).

*Composition and Properties of Fraction A (Nucleoprotein)*—Fraction A, as already stated, is obtained by precipitation with acetic acid at pH 4.2 to 4.4. The white, flocculent precipitate is readily and completely soluble at neutral reaction, but only in the presence of salt. The solutions do not coagulate on heating at neutral reaction and in the presence of electrolytes.

Samples of the fractions obtained from different batches of culture were dried over  $P_2O_5$  in a high vacuum and were then analyzed for nitrogen and phosphorus.

The nitrogen content was found to vary from 14.15 to 15.50 per cent and the total phosphorus from 3.79 to 4.40 per cent. The nitrogen to phosphorus ratios varied from 3.22 to 4.09. It may be pointed out at this time that no detectable inorganic phosphorus is released into solution in the course of the change from the Gram-positive to the Gram-negative state. Solutions of Fraction A give strongly positive biuret and Sakaguchi reactions, but the Millon and glyoxylic acid tests are negative. After boiling with hydrochloric acid for 2 to 3 minutes a positive Bial's test for pentoses is obtained, and the solution reduces Benedict's reagent. The nitroprusside reaction for sulfhydryl groups is negative, even when carried out on a sample of dry material to increase the sensitivity (15). The barium sulfate test gave, however, a very faint opalescence when carried out on a large amount of material. The presence of purine bases in the molecule was established by a positive murexide test. No test for pyrimidines was carried out.

These properties of Fraction A, when considered jointly, lead one to the conclusion that Fraction A, or at any rate the major part of it, consists of a nucleoprotein. The ultraviolet absorption spectrum was therefore examined, and it was found that the solutions exhibit a broad band in the region characteristic for nucleic acid. The spectroscopic analyses are described in a separate publication (16). The ultraviolet absorption spectrum, however, did not show any bands corresponding to the aromatic amino acids, a fact which bears out the negative Millon and glyoxylic acid reactions.

It will be noted that this nucleoprotein fraction has not been purified and that the above analyses have been carried out on a crude acetic acid precipitate obtained at pH 4.2; it is therefore possible that other proteins, mucoproteins for example, also precipitable at this pH, might be present as impurities. Some of the acetic acid precipitate was therefore brought to pH 2.5 with HCl, and shaken at this pH, in the hope of causing contaminating mucoproteins to go into solution. It was found, however, that no protein could be detected in the HCl supernatant obtained in this manner from Fraction A.

*Effect of Repeated Extraction of Cells at Neutral Reaction*—The data presented above deal with the fractions released into solution during a single and relatively short period of incubation at pH 7.0 and at 37°. The following experiment shows the effect of prolonged incubation on the nature of the material released into solution and precipitable with acetic acid.

80 liters of a 4 hour culture of pneumococci killed with acetic acid by the technique described were resuspended in saline, adjusted to pH 7.0, and incubated at 37°. Over 75 per cent of the cells had become Gram-negative after 3 hours incubation; the cells were then centrifuged off and the filtered supernatant precipitated with acetic acid (first extract). The cells were resuspended at pH 7.0, incubated for another 24 hours, again centrifuged, and the filtered supernatant precipitated with acetic acid (second extract). The process was repeated (a further 48 hours incubation) but the third acetic acid precipitate was so small that it was impossible to determine its dry weight.

The first extract (3 hours incubation) yielded 287.1 mg. of an acetic acid precipitate containing 14.15 per cent nitrogen and 4.40 per cent phosphorus (N:P ratio 3.22). The second acetic acid precipitate, obtained after 27 hours incubation, weighed 95.2 mg. and contained 14.41 per cent nitrogen and 3.97 per cent phosphorus (N:P ratio 3.65). The third acetic acid precipitate obtained after 75 hours incubation weighed less than 10 mg.; the N:P ratio was 22.65.

This third extract appears, therefore, of a different nature from the material which constituted the totality or the majority of the first and second extracts. Biological tests afford additional evi-

dence that the first and the third extracts are different in nature. Fraction A (corresponding to the first extract) has been injected into mice in amounts up to 10 mg. without giving any evidence of purpuric reaction; on the contrary mice receiving even much smaller amounts of the third extract all developed purpura within 18 hours.

*Composition and Properties of Fraction B (Nucleic Acid)*—As described earlier in this paper, Fraction B was obtained as a precipitate by the addition of HCl to pH 2.5 to the acetic acid-soluble fraction of the material released into solution when the pneumococci became Gram-negative.

TABLE I

*Nitrogen and Phosphorus Content of Fraction B (Precipitated with HCl)*

Experiment No.	Weight of fraction	Total nitrogen	Total phosphorus	N:P ratio
	mg.	per cent	per cent	
2	40.3	13.90	6.36	2.18 (Unpurified)
4	128.9	15.20	7.05	2.16 "
6	62.6	13.89	7.75	1.79 (Reprecipitated)
7	345.6	14.05	7.06	1.99 "
8	311.6	13.75	7.78	1.77 "

In Experiments 2 and 4 the crude HCl precipitate was analyzed for nitrogen and phosphorus after desiccation in a high vacuum over phosphorus pentoxide, and then in a vacuum oven at 80°. In Experiments 6, 7, and 8, the HCl precipitate was freed from traces of nucleoprotein by redissolving in a small volume of water and precipitating any nucleoprotein present with acetic acid at pH 4.3. This precipitate was centrifuged off, and the supernatant reprecipitated with HCl at pH 2.5, giving Fraction B<sub>2</sub> which was dried with alcohol and ether. The final Fraction B<sub>2</sub> was a white powder; when precipitated with hydrochloric acid the dry powder was readily soluble in distilled water on the addition of a little alkali; after precipitation with alcohol it was immediately soluble in distilled H<sub>2</sub>O, giving a perfectly clear solution. The nitrogen and phosphorus analyses are given in Table I.

The material obtained from Experiments 6, 7, and 8, in Table I, was biuret-free in each case. The solubility properties and the

high phosphorus content, together with the nitrogen to phosphorus ratio, particularly in the reprecipitated samples, suggest that the substance is closely related to nucleic acid.

The hydrochloric acid precipitate in Experiment 8 was again dissolved in water and reprecipitated with alcohol. The resulting precipitate, after being dried with alcohol and ether, was again analyzed for nitrogen and phosphorus; these analyses made on the sodium salt of the nucleic acid are shown in Table II, together with the figures calculated for the free nucleic acid and those found for the free acid.

It will be seen from Table II that Fraction B<sub>2</sub> gave very constant values for nitrogen and phosphorus even after a further reprecipitation with alcohol.

TABLE II  
*Nitrogen and Phosphorus Content of Fraction B<sub>2</sub> (Nucleic Acid)*

	Total nitrogen	Total phos- phorus	N:P ratio
	<i>per cent</i>	<i>per cent</i>	
Pptd. with alcohol as Na salt.....	13.04	7.26	1.79
Calculated from these figures as free acid.....	13.92	7.75	1.79
Found as free acid.....	13.75	7.78	1.77

Owing to the small amounts of material available it has not been possible to carry out a complete identification of the constituent substances after hydrolysis, but much information has been obtained from various reactions, the results of which, when considered together, show that Fraction B<sub>2</sub> consists of ribonucleic acid.

A solution containing 3.3 mg. per cc. gave negative biuret, Sakaguchi, and Millon reactions. A solution containing 4.14 mg. per cc. was hydrolyzed by heating with 2 per cent H<sub>2</sub>SO<sub>4</sub> in a water bath under a reflux for 2 hours. The solution began to clear almost immediately on heating and at the end of 2 hours a clear hydrolysate was obtained. The hydrolysate was made up to a volume corresponding to 3 mg. of original substance per cc. This acid hydrolysate was found to reduce Benedict's solution, and to give an intense Molisch reaction. When it was heated with Bial's reagent, an intense green color and green precipitate were produced; in view of the fact that Fraction B<sub>2</sub> was obtained from rough

pneumococci, *i.e.* cells devoid of capsular polysaccharide, and in view of the method of preparation of the fraction and of the intensity of the reaction with Bial's reagent, it seems highly probable that the positive reaction indicates the presence of a pentose rather than a uronic acid; the nucleic acid therefore appears to belong to the ribose or yeast type. To confirm this the Feulgen reaction (13) with Schiff's reagent was carried out on a sample of the substance, the technique described by Widström (14) being used; the reaction was found to be completely negative, indicating the absence of desoxyribose. The presence of purine bases in the molecule was established by a positive murexide test. The ultraviolet absorption spectrum of neutral solutions of the sodium salt of the nucleic acid has also been examined, and compared with the absorption of solutions of equivalent concentration of yeast nucleic acid. Both substances showed a powerful absorption with a maximum in the region of 2600 Å. and a minimum in the region of 2300 Å. The agreement between the absorption curves of these two solutions is discussed in a separate communication (16). A further point suggesting that this nucleic acid is of the ribose type is the fact that it is precipitated by glacial acetic acid as well as by hydrochloric acid.

*Action of Pancreatic Ribonuclease on Fraction B<sub>2</sub>*—A heat-resistant enzyme capable of decomposing ribonucleic acid with a high degree of specificity, has recently been isolated from pancreatic extracts (17). The action of this enzyme on Fraction B<sub>2</sub> was tested as follows:

0.5 cc. of a solution of nucleic acid (Fraction B<sub>2</sub>) containing 4.17 mg. per cc. was added to four tubes. The ribonuclease was added to three of the tubes in amounts of 0.1, 0.01, and 0.001 mg. respectively; the fourth tube was kept as control. Phosphate buffer at pH 7.0 was added to give a final volume of 2 cc. and the tubes were incubated at 59° for 2 hours. At the end of this time, 1 N HCl was added to test for the decomposition of the nucleic acid.

It was found that 0.001 mg. of the enzyme had been sufficient to decompose the 2 mg. of nucleic acid. In view of the specificity of the enzyme and, in particular, of the fact that it does not attack thymus nucleic acid, this experiment establishes once more that Fraction B<sub>2</sub> is a nucleic acid of the ribose type.

*Quantitative Relationships between the Nucleic Acid and Nucleo-*



*protein Fractions*—Although it has been found impossible to obtain quantitative recoveries of the two fractions (A and B) released in solution when pneumococci change from the Gram-positive to the Gram-negative state, it appears that on the average each one of the two fractions corresponds to 2 to 5 per cent of the dry weight of the cells. However, it was also observed that the amount of nucleic acid recovered did not bear a constant ratio to the amount of nucleoprotein; in fact, it was a constant finding that when the yield of nucleoprotein was low, the yield of nucleic acid was high. In one experiment, for instance, in which the cells had been killed at pH 4.0 and the change from Gram-positive to Gram-negative had been very slow (72 hours), only a trace of nucleoprotein was released, whereas the yield of nucleic acid was exceptionally high.

#### DISCUSSION

Pneumococci killed with acetic acid at pH 4.2 to 4.4 retain their Gram-positive character as long as they are maintained at this acid reaction; if, however, the acid-killed cells are then resuspended in a neutral medium, there takes place an enzymatic reaction which changes the cells from the Gram-positive to the Gram-negative state, and releases into solution both a nucleic acid and a nucleoprotein. Evidence for the enzymatic nature of this reaction will be presented in a separate communication.

Owing to the small amounts of material available, isolation of the constituents of the nucleic acid has not been carried out. However, convincing evidence has been obtained that the nucleic acid belongs to the ribose, or yeast type. It will have been noted that the preparations obtained, although they were protein-free, did not give exactly the same N:P ratio as yeast nucleic acid; it should be remembered in this connection that yeast nucleic acid is not the only ribose nucleic acid known, since Jorpes (18), for example, isolated from pancreas a ribose pentanucleotide with an N:P ratio of 1.80.

The nucleoprotein fraction has not been studied extensively, as most of the material obtained has been used for the immunization of experimental animals. The following points, however, have been established. The nucleoprotein is characterized by a high phosphorus content (4 per cent), suggesting that the protein with which the nucleic acid is combined is of a relatively low

molecular weight. The protein appears to be deficient in aromatic amino acids, and possibly in sulfur, and fails to coagulate on heating. These properties, when considered jointly, suggest that the protein is of the protamine or histone type, as in the nucleoproteins isolated from fish sperm and other materials (19).

It is commonly stated that all bacterial cells give a positive Feulgen reaction (20) and therefore contain a desoxyribose (thymus type) nucleic acid. Indeed Schaffer, Folkoff, and Bayne-Jones (5) obtained from *Escherichia coli* a protein-free compound rich in phosphorus, which they regarded as nucleic acid, and which must have belonged to the desoxyribose group, since they failed to get a test with the orcinol reagent. Johnson and Brown (4) isolated from tubercle bacilli a nucleic acid—tuberculinic acid—which also belonged to the desoxyribose type. We have found that pneumococci which have been rendered free of ribose nucleic acid by the enzymatic technique described in this paper still give a powerful test with Feulgen's reagent; however, we have so far failed to obtain thymus nucleic acid from pneumococci, or to demonstrate its presence by spectroscopic analysis. In view of the lack of specificity of Schiff's reagent (14, 21) it seems hardly justifiable to consider that a positive Feulgen test is sufficient evidence of the presence of desoxyribose nucleic acid in pneumococci—or in other bacterial cells.

The results of Heidelberger and Kendall (9) leave little doubt of the existence of ribose nucleic acid in hemolytic streptococci. It has been shown that the same substance is an important constituent of the pneumococcus cell. Finally, we have found that it is possible to release from staphylococci, by an enzymatic technique similar to the one used in the present study, a substance which gives the tests of ribose nucleic acid. It is of interest that this same type of nucleic acid is present in three different species of Gram-positive cocci. A ribonucleic acid has also been isolated from *Mycobacterium phlei* (7); its nitrogen and phosphorus contents are strikingly similar to those of the pneumococcal nucleic acid described here.

#### SUMMARY

Nucleic acid and a nucleoprotein have been recovered from the material released into solution from pneumococcus cells by a

controlled process of autolysis which does not involve disintegration of the cell structure. The nucleic acid belongs to the ribose type. The protein moiety of the nucleoprotein appears to belong to the group of protamines or histones.

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## THE ULTRAVIOLET ABSORPTION SPECTRA OF FRACTIONS ISOLATED FROM PNEUMOCOCCI

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It has been shown (1) that pneumococci can be converted from the Gram-positive to the Gram-negative state under conditions that do not involve a disintegration of the cell bodies. In the course of this change in staining reaction there are released into solution, by enzymatic action, certain substances, two of which have been investigated. The isolation and the chemical nature of these substances are described in another paper (2). It may be stated here, however, that one of the fractions, which is obtained by precipitation with acetic acid at pH 4.2, consists of a nucleoprotein. After removal of this nucleoprotein from the solution, further acidification with hydrochloric acid to pH 2.5 brings about the precipitation of a second substance, which has been identified as a nucleic acid containing about 14 per cent of nitrogen and 8 per cent of phosphorus.

Caspersson (3) has reviewed the literature concerning the ultraviolet absorption spectra of nucleic acids. These substances exhibit a very strong absorption band in the region 2500 to 2700 Å. It was decided, therefore, to investigate the ultraviolet absorption spectra of the fractions described above.

### EXPERIMENTAL

*Material*—The culture used in this work was an R strain of pneumococci derived from Type II. The preparation of the bacterial suspensions has been described in a previous communication (1).

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The bacterial cells were separated from the medium by centrifugation, washed once, and resuspended in 0.1 M acetate buffer (pH 4.2). They were incubated for 12 hours at 37° at this pH, and were then centrifuged and resuspended in a dilute buffer solution at pH 7.0, at which reaction the cells became Gram-negative. The suspension was then centrifuged and the supernatant filtered through a Berkefeld filter (V). The spectroscopic measurements described below were carried out on the supernatant and on the nucleoprotein and nucleic acid fractions obtained from it.

A suspension of pneumococci of the same concentration as that used in the preparation of the Gram-negative cell bodies was incubated at pH 4.2 for the same length of time as that required to cause the cells resuspended at pH 7.0 to become Gram-negative. The cells maintained at pH 4.2, which had remained uniformly Gram-positive, were then centrifuged, and the neutralized, filtered supernatant was used as a control for spectroscopic examination.

*Spectroscopic Methods*—The absorption was measured with the aid of a Spekker spectrophotometer and a small Hilger quartz spectrograph. The curves were obtained by plotting the absorption coefficient against the wave-length in Ångström units. The absorption coefficient,  $\alpha$ , is defined by the expression  $\alpha = 1/cl \log I_0/I$  where  $c$  is concentration in mg. per cc.,  $l$  is the cell length in cm.,  $I_0$  is the intensity of light falling on the cell, and  $I$  is the intensity of light after passing through cell thickness (1 cm.)  $l$ . Since previous work (4) has shown that weak, diffuse bands are not readily seen with the photometer and line source, the curves were checked by means of photographs taken with the continuous light from a hydrogen discharge tube.

### Results

On spectroscopic examination the filtered solution in which the pneumococci had been allowed to become Gram-negative was found to contain a substance or substances showing a broad, diffuse band in the region 2500 to 2700 Å. On the other hand, the filtrate from the suspension in which the cells had remained Gram-positive showed no absorption in this region.

The absorption curve of a solution containing 0.128 mg. per cc. of the nucleoprotein is shown in Fig. 1 (Curve C). The absorption maximum at 2600 Å. cannot be ascribed to tryptophane,

which displays a maximum at 2780 Å., or to tyrosine, which has a maximum at 2750 Å. (5). However, it corresponds closely to the maxima displayed by the nucleic acids from pneumococcus (Curve A) and from yeast (Curve B). In an early experiment on

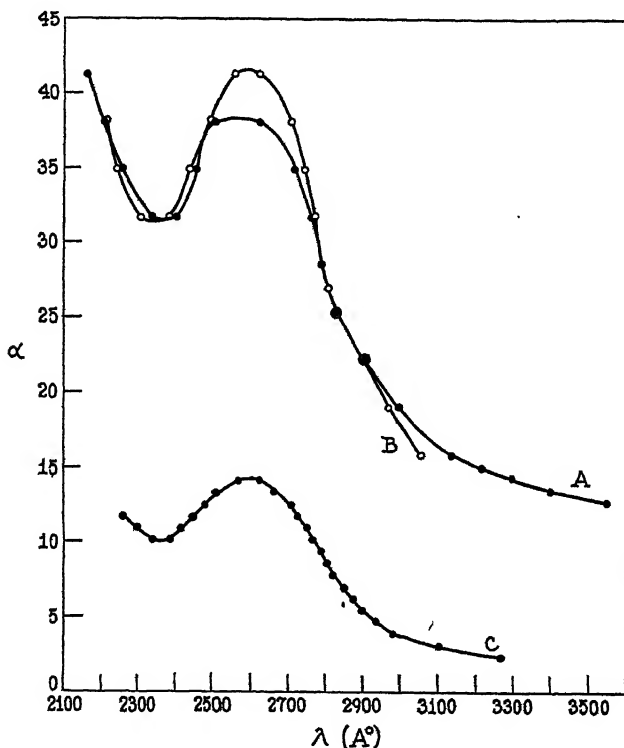


FIG. 1. Absorption coefficient curves. Curve A, sodium salt of nucleic acid obtained from pneumococcus (0.031 mg. per cc.); Curve B, yeast nucleic acid (0.031 mg. per cc.); Curve C, acetic acid precipitate (nucleo-protein) obtained from supernatant in which pneumococci had become Gram-negative (0.128 mg. per cc.).

an impure preparation there was present a band which was thought to be due to tryptophane; but in all subsequent experiments in which the conditions for the release of the nucleoprotein from the cell were more carefully controlled, so that no disintegration of the cell bodies could be observed, this band was no longer discernible.

## DISCUSSION

When pneumococci, killed with acetic acid, are allowed to become Gram-negative at neutral reaction, they release into the medium substances which exhibit a broad, diffuse absorption band in the region 2500 to 2700 Å. The position of this band agrees with that of nucleic acid. The two acid-precipitable fractions obtained from the cell-free filtrates of the bacterial suspensions also absorb in this region; moreover, chemical studies (2) on these two fractions had led to the view that they consisted respectively of a nucleoprotein and a nucleic acid.

Although the nucleoprotein solutions absorb in the region characteristic of nucleic acid, no bands were found at wave-lengths associated with the presence of the aromatic amino acids (4); this finding is in agreement with the fact that these solutions do not give the Millon or glyoxylic acid reactions. It may be mentioned in this respect that not only does the nucleoprotein fail to reveal any bands characteristic of aromatic amino acids, but these bands are also absent from the whole solution in which pneumococci had changed from the Gram-positive to the Gram-negative state; it appears likely, therefore, that if proteins containing aromatic amino acids are released into solution during this change in staining reaction they must be present only in very small amounts.

## SUMMARY

1. Spectroscopic examination has been carried out on the substances released into solution when pneumococci are converted from the Gram-positive to the Gram-negative state.

2. The absorption spectra, in conjunction with chemical analyses, have established the presence of a nucleic acid and a nucleoprotein among the substances.

3. The nucleoprotein fractions exhibit no band absorption in the region characteristic of aromatic amino acids.

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## STUDIES ON KETOSIS

### XV. THE COMPARATIVE METABOLISM OF *d*-MANNOSE AND *d*-GLUCOSE\*

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Despite the fact that mannose may be classed as a physiological sugar, the information regarding its metabolism is quite fragmentary. Not only is it found as a polysaccharide in the seeds of the tagua palm, but more recent investigations point to it as a component of the glycoprotein ovomucoid (1, 2) as well as of the serum proteins of horse plasma (3). Most investigators agree that it is quite slowly absorbed in distinction to galactose and glucose. The ratio of absorption of 33 of *d*-mannose compared with *d*-glucose as 100, obtained by Wilbrandt and Laszt (4), is somewhat higher than the earlier values reported by Cori (5) of 19 or the more recent ones of Westenbrink (6) of 15. In distinction to glucose its absorption proceeds without phosphorylation by pure diffusion (Verzár (7)) and somewhat more rapidly at higher concentrations. Sunaba (8) reports that mannose is a far less effective source of glycogen than glucose.

In the present experiments we have first attempted to evaluate the absorption coefficient of mannose in view of the contradictory evidence of several investigators. Secondly, we have compared the glycogenic ability of this monosaccharide with glucose and have made a comparison of its ketolytic activity with the latter hexose.

\* These data were presented before the meeting of the Society for Experimental Biology and Medicine, Southern California Section, May 2, 1938. This work was assisted by a grant from the Rockefeller Foundation.



*Procedure*

Studies on absorption and glycogen formation were made at 2, 4, and 6 hour intervals after the first administration of the sugars by stomach tube. Sugar was fed at 2 hour intervals in sufficient amount to allow maximum absorption. In a second series of tests comparisons were made of the glycogen deposition at similar intervals when doses of glucose comparable to the average mannose absorbed<sup>1</sup> were administered hourly. At the appropriate times the animals were sacrificed, sodium amytal being used as the anesthetic. Absorption was determined by the Cori method (5) and glycogen by the procedure of Good, Kramer, and Somogyi (9).

The comparison of ketolytic effect of glucose and mannose was made on the endogenous ketonuria of rats by the method described earlier (10). Acetone bodies were estimated by the Van Slyke procedure, while the usual Kjeldahl method was employed for urine nitrogen. Liver water and liver fat were determined as described earlier (10).

To determine the mannose or glucose content remaining in the gut, the usual Shaffer-Hartmann method was used on the filtrate following precipitation of the proteins by  $\text{ZnSO}_4$  and  $\text{NaOH}$ , according to Somogyi. Contrary to the report of Moore, Lloyd, and Burget (11), we have found that this procedure is entirely reliable for mannose. In five series of tests carried out in triplicate by three different observers with mannose solutions of 0.962, 0.721, 0.481, and 0.240 mg. of sugar per 5 cc. respectively (prepared from a stock solution in which the concentration was determined polariscopically), an average of 87 per cent of the reduction of glucose was obtained. The average reductions compared with glucose at these dilutions were 85.9, 88.2, 85.8, and 88.2 per cent respectively. Moreover, we have had no difficulty in getting consistent checks with the gut washings containing mannose. c.p. mannose of Coleman and Bell was employed. The specific rotation was  $13.72^\circ$ , compared with a theoretical value of  $14.25^\circ$ . Commercial cerelese was used for the source of glucose in which

<sup>1</sup> Owing to an error in the calculation of the amount of mannose absorbed, the dose of glucose administered was only 87 per cent of the level of mannose fed.

the purity was determined by reduction and by polariscopic examination.<sup>2</sup>

Male rats from our stock colony were used for the determinations of glycogen and absorption following a 48 hour fast. Females were employed in the ketosis studies. In the latter tests the glucose and mannose were administered in 10 per cent NaCl solution, in three equal divided doses at 8 hour intervals (instead of two doses) to avoid any chance of a mannosuria due to too large a dose of this component at one time. No excretion of mannose was found in the urine under these conditions.

TABLE I  
*Comparative Absorption of Glucose and Mannose in Male Rats*

Sugar administered	Duration of tests									Average of all tests†
	2 hrs.			4 hrs.			6 hrs.			
	Body weight	No. of experiments	Absorption coefficient*	Body weight	No. of experiments	Absorption coefficient*	Body weight	No. of experiments	Absorption coefficient*	
	gm.			gm.			gm.			
Glucose.....	176	4	127	192	4	126	186	4	145	135 ± 2.6
" (12)...	186	5	128	192	6	117	169	6	157	
Mannose.....	167	9	17.6	173	8	16.9	174	7	14.7	16.5 ± 0.7

\* The absorption coefficient is given in mg. of sugar per 100 gm. of rat per hour.

† Including the probable error of the mean calculated from the standard deviation.

### Results

The results of the absorption tests with glucose and mannose are given in Table I, while the summary of glycogen studies is reported in Table II. The averages on glucose are practically identical with values reported earlier (12). Table III summarizes some experiments in which the sugars were given intraperitoneally. The results of the ketolysis tests are summarized in Table IV.

<sup>2</sup> The authors wish to thank H. D. Blunden for carrying out these estimations.

TABLE II

*Comparative Glycogen Formation in Liver of Male Rats after Mannose and Glucose*

The glycogen values are given in per cent. The numbers in parentheses are the total number of experiments in the group.

Sugar administered	Glycogen controls	Glycogen deposited after sugar administration*		
		2 hr. test	4 hr. test	6 hr. test
Glucose.....	0.21 (11)	1.71 (4)	2.99 (4)	4.83 (4)
" †.....	0.59 (15)	1.57 (10)	3.05 (10)	4.54 (10)
Mannose.....	0.20 (17)	0.30 (8)	0.64 (10)	0.53 (10)
Glucose (13.4 mg. per 100 gm. per hr.).....	0.19 (6)	0.19 (6)	0.76 (6)	1.31 (6)

\* Control values have been deducted from these averages.

† Data from Deuel, Hallman, Murray, and Samuels (12).

TABLE III

*Comparative Glycogen Formation in Male Rats 6 Hours after Intraperitoneal Injection of Glucose and Mannose*

Six experiments were carried out with each sugar.

Sugar injected	Body weight	Dose	Glycogen	Urinary excretion
	gm.	mg.	per cent	mg.
Glucose.....	166	300	1.76	
Mannose.....	167	345	1.28	124

TABLE IV

*Comparative Ketolytic and Protein-Sparing Action of Glucose and Mannose on Endogenous Ketonuria of Rats*

Mannose was fed at a level of 57.5 mg. per 100 sq. cm. and glucose in the amount of 50 mg. per 100 sq. cm.

Sugar administered	Average body weight	Total No. of experiments	Acetonuria, mg. per 100 gm. rat				Urine nitrogen, mg. per 100 gm. rat			
			2nd day	3rd day	4th day	Average of all tests†	2nd day	3rd day	4th day	Average of all tests*
	gm.									
Fasting controls.....	171	23	45.4	65.9	46.2	52.8 ± 2.2	45.5	40.4	36.8	41.2 ± 0.8
Mannose.....	178	22	36.5	53.4	41.7	44.4 ± 1.7	44.4	37.9	30.0	37.1 ± 1.4
Glucose.....	165	24	16.2	25.3	16.6	19.3 ± 2.8	44.3	36.6	31.1	37.4 ± 1.1

\* Including the probable error of the mean calculated from the standard deviation.

## DISCUSSION

The extent of absorption of mannose which we have noted is lower than the values previously found by other investigators. The average absorption coefficient of the twenty-nine experiments on glucose reported in Table I is 134 mg. per 100 gm. of rat per hour, while the corresponding mean of the twenty-four tests on mannose is 16.5 mg. Considering the rate of glucose absorption as 100, this gives a comparative value for mannose of 12.2, which is slightly lower than the results of Westenbrink and Cori and only approximately one-third of the proportion given by Wilbrandt and Laszt. On the absolute basis the rate of mannose absorption (16.5 mg. per 100 gm. of rat) is only about 50 per cent of the result obtained by Cori (5) of 34 mg.

Mannose is able to form small amounts of glycogen. However, the maximum percentage of glycogen apparently is formed within the 4 hour interval and no increase over this level was to be noted in the 6 hour tests. This is in distinction to the results obtained on glucose in which an increase in glycogen continues during maximum absorption, at least to the 8 hour period (12).

That the superior glycogenic activity of glucose is not entirely to be ascribed to its increased rate of absorption is indicated by the fact that the glycogen level in the liver is also higher when glucose is administered in hourly doses at a slightly lower rate than that at which the mannose could be absorbed. Thus, although the levels of the glucose controls approximated that of the mannose experiments in the 2 and 4 hour tests, the glycogen deposition was much higher in the 6 hour group. This demonstrates that glucose is definitely a better glycogen former when it is absorbed at the same rate as the mannose.

The comparison of the ketolytic activity of the two sugars is of especial interest. When glucose was fed at a level of 50 mg. per 100 sq. cm., the average level of endogenous ketonuria was lowered from 52.8 mg. per 100 gm. of rat to a mean of 19.3 mg. The administration of a somewhat larger dose of mannose (57.5 mg. per 100 sq. cm.) only slightly depressed the excretion of ketone bodies. The average level in the latter case was 44.4 mg. It is evident that mannose differs from the other physiological sugars, galactose and fructose, in this respect. Both galactose and fructose have

been shown to be superior to glucose in lowering the fasting ketonuria of man (13) and in the ketonuria of dogs following a high fat diet (14), while galactose was also shown to have a greater effect on the exogenous ketonuria of rats (15). On the other hand, the nitrogen-sparing action of glucose and mannose is only slightly greater in the former case.

#### SUMMARY

Mannose is absorbed by male rats at a rate of 12.3 compared with glucose as 100. In absolute values, the absorption coefficient was found to be 16.3 mg. per 100 gm. of rat.

Small amounts of glycogen were deposited in the liver after mannose, but the glycogen formation was much lower than after glucose even when the latter sugar was given in an amount comparable to the rate of mannose absorption.

When fed to fasting rats having an endogenous ketonuria produced by a previous high fat diet, mannose only slightly lowered the existing ketonuria, while glucose depressed it to about one-third of its original level.

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# A METHOD FOR THE DETERMINATION OF NICOTINIC ACID, NICOTINAMIDE, AND POSSIBLY OTHER PYRIDINE-LIKE SUBSTANCES IN HUMAN URINE\*

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The discovery of nicotinic acid in rice bran (1) and in yeast (2, 3) directed attention of biochemists to the natural occurrence of this pyridinecarboxylic acid. Funk suggested that it had some relation to vitamin B, but subsequent investigation showed that it made no part of vitamin B<sub>1</sub> or B<sub>2</sub>. The discovery by Warburg and associates (4) that it was present as the amide in coenzyme and by von Euler *et al.* (5) that it was a part of cozymase revived interest in it and indicated that it was playing an important part in the respiratory metabolism of both animal and plant cells. Following these clues, Elvehjem and his associates (6) discovered that its administration would cure the disease of black tongue in dogs, and Spies, Cooper, and Blankenhorn (7) reported its favorable action in the treatment of human pellagra, an action confirmed by others (8).

Since there is no evidence that pyridine can be formed in the human body, it is evident that nicotinic acid or its amide must be an important article of human diet and that it is apparently necessary for the normal health of the mucosa of the mouth and intestine, nervous system, and skin, since these all are impaired in pellagra and improved by the ingestion of nicotinic acid or the amide. It must be necessary for the muscles also, and probably for the liver and some other organs, since it is an essential

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part of cozymase and of the coenzyme (Warburg *et al.*) of these tissues and organs.

These facts made it desirable to discover a method by which its presence in animal tissues, fluids, and secretions could be detected and the amount present determined. The pressing need for the discovery of a method making it possible to detect a prepellagrous condition, before the well marked skin and other changes were so developed as to make diagnosis easy, led to the first efforts being directed toward a method for measuring the amount in the urine both in normals and in subjects with dietary deficiency diseases, such as pellagra. Such a method was found, as announced in a preliminary communication, and showed at once that pellagrins when untreated, and normal people, when on a diet deficient in nicotinic acid, had no substances in the urine which would develop the color reaction of the method. They excreted no nicotinic acid conjugates, at least in a form which would give the reaction described herein.<sup>1</sup>

An investigation of the literature revealed a characteristic color reaction for pyridine and certain of its homologues. It was first described by Vongerichten (10), and was developed by Zincke (11, 12), König (13), Reitzenstein (14), Baumgarten (15), and others. The reaction involved the addition of 2,4-dinitrochlorobenzene to the tertiary nitrogen of the pyridine ring. This addition product was decomposed by amines, such as aniline, or by alkali hydroxides, to yield a deeply colored product. Further study by König showed that cyanogen bromide gave a similar addition product with pyridine and that decomposition by aniline or by alkalies yielded similar soluble colored products. The opinions in regard to the identity of these colored substances have been controversial, but the most reliable evidence indicates that the derivatives are open chain compounds with conjugated double bonds, and that they are related to glutaconic dialdehyde (15). It was decided to try this reaction upon nicotinic acid, the  $\beta$ -monocarboxylic acid of pyridine, and upon certain other related compounds such as the true salts and conjugated derivatives, and nicotine itself, since a study of the literature did not reveal any

<sup>1</sup> After this paper had been submitted for publication, practically the same method was published by Karrer and Keller for the determination of nicotinamide in cozymase (9).

application of this reaction to substances other than the pyridine-like bases.

It was soon observed that free nicotinic acid, the sodium salt, and the amide would not give a color with 2,4-dinitrochlorobenzene and alkali at the temperatures 60–70° and in conditions used in previous work with pyridine and other like bases as described by the authors cited. However, it was noticed that in the absence of a solvent, nicotinic acid and the above reagent fused at a low temperature, between 85–100°, depending upon the proportion of each. After this fusion, the addition of an alcoholic solution of alkali to the residue gave a brilliant purple color. Since this color was similar to the purple pyridine derivative, it was thought at first that the color was due to some pyridine formed by the fusion. Subsequent work, however, showed this to be improbable, since cyanogen bromide, which reacted with the pyridine, did not yield a colored derivative of nicotinic acid under any of the conditions which have been tried.<sup>2</sup> Furthermore, in an attempt to extract the colored compounds with organic solvents, it was observed that although the colored pyridine derivative was easily extracted with isopropyl and other higher alcohols from the alkaline liquid, the purple derivative of nicotinic acid had a greater affinity for the aqueous alkali layer and would exhibit a partition in the two solvents dependent upon the concentration of the alkali present. The greater the concentration of the alkali, the less went into the alcohol. This gave evidence that the colored substance was acidic and that the carboxyl group remained intact during the reaction with 2,4-dinitrochlorobenzene. The two derivatives, *i.e.* from pyridine and from nicotinic acid, were evidently not identical. Hence the color was not due to pyridine formed from nicotinic acid as had at first been supposed.

At present, after a study of the factors necessary to produce this color on a quantitative basis, it is believed that an addition compound similar to that described by Zincke (11) for pyridine is formed when an alcoholic (95 per cent) solution of nicotinic acid and 2,4-dinitrochlorobenzene is evaporated to dryness at 95–105°. The fusion and reaction are complete within 10 minutes

<sup>2</sup> Since the paper was submitted, the successful use of cyanogen bromide to produce a colored derivative of nicotinic acid has been described by Swaminathan (16).



at 100–105° after the solvent is evaporated. The addition product is yellowish but develops red color on the addition of alkali. When only a slight excess of the reagent, 2,4-dinitrochlorobenzene, is used, and the colored solutions are diluted to a fixed volume with more of the cold alcoholic sodium hydroxide, the depth of color produced is proportional to the quantity of nicotinic acid or amide taken. The amount of light absorption of the color can then be successfully reproduced for different concentrations of nicotinic acid in the range of 0.1 to 0.5 mg. on the Sheard-Sanford photometer,<sup>3</sup> and the amount of nicotinic acid or the amide thus determined. In this photometer colored filters, a photoelectric cell, and a sensitive microammeter are used. Once calibrated for the colored substance under investigation this photometer is very convenient. Still smaller amounts could no doubt be determined, but with less accuracy with this instrument.

Nicotinic acid itself is not the only substance giving the color. Other compounds related to it which have been tested are nicotinamide, sodium nicotinate, coramine (diethyl nicotinamide), trigonellin, picolinic acid,  $\alpha$ -picoline, and nicotine. The sodium salt of nicotinic acid gives a purple color as the free acid does. The betaine, trigonellin, gives no color; *i.e.*, a negative test. The amide gives a Burgundy red, a yellow-red in dilute solutions; but the substituted amide, coramine, again gives a purple color. Why the substitutions of ethyl groups for the hydrogen atoms of the amide nitrogen should shift the absorption toward the red is not yet understood. Picolinic acid, the  $\alpha$ -monocarboxylic acid of pyridine (m.p. 136°, uncorrected), does not give a colored product under the same conditions. However, a color does develop after the acid is heated with the reagent over a free flame.<sup>4</sup> The odor of pyridine is then apparent. The closely related base,  $\alpha$ -picoline, *i.e.*  $\alpha$ -methylpyridine, gives a purple color under the same conditions as pyridine (4) or nicotinic acid, so the "hindrance" to the reaction in picolinic acid due to the carboxyl group in the  $\alpha$  position is of interest. The picolinic acid-glycine conjugate, kindly supplied us by Mr. Birnbaum, also failed to develop color under the conditions of the nicotinic acid reaction. Nicotine

<sup>3</sup> Obtained from the Central Scientific Company of Chicago.

<sup>4</sup> This fact was communicated to us by Mr. S. M. Birnbaum who is working on the excretion of picolinic acid.

itself, however, gives a purple color. This fact should be useful, since tests for this alkaloid are not numerous.

The reaction is, then, sufficiently selective and delicate to enable one to make a quantitative estimation of very small

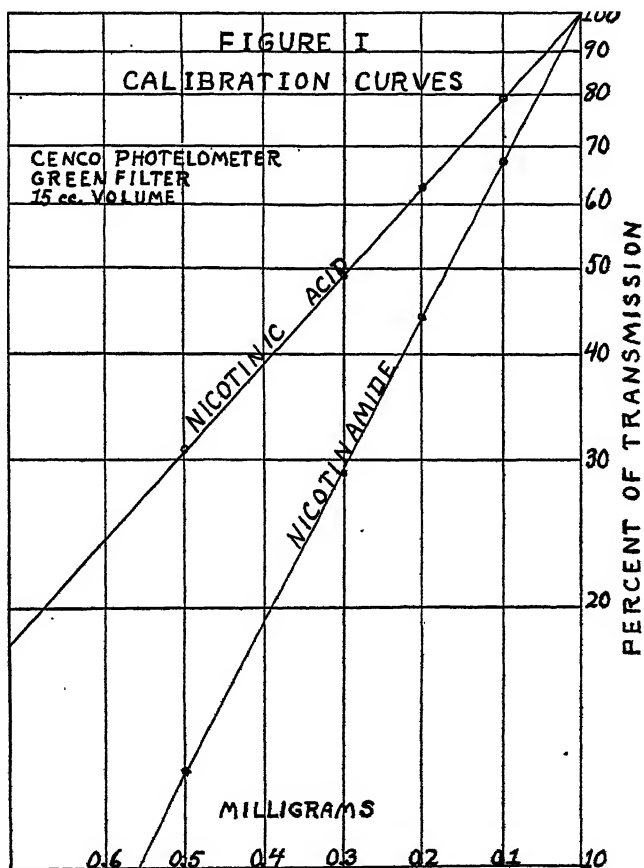


Fig. 1. Calibration chart of nicotinic acid and amide

amounts of nicotinic acid or amide in the absence of other pyridine

The method developed was the following: The photelometer is first calibrated by the use of solutions of the acid or amide of known strength, amounts of solution being taken containing

between 0.1 and 0.5 mg. The calibration curves thus obtained with the green filter of the photometer are given in Fig. 1.

It will be seen from Fig. 1 that for quantities between 0.1 and 0.5 mg. the points when plotted on logarithmic paper with the per cent of transmission of the light, as read from the photometer, as ordinates, fall along straight lines, but that the line of the amide differs from that of the acid.

The standard solutions used to determine the points in Fig. 1 were treated in the following way: 1, 2, 3, and 5 cc. samples of aqueous solutions containing 10 mg. of nicotinic acid in 100 cc., and others containing 10 mg. of nicotinamide in 100 cc., were pipetted into 30 cc. beakers; the solutions were evaporated cautiously at 80–100° or on the water bath and were removed as soon as the solvent had evaporated. To each beaker was then added 1.0 cc. of a 1 per cent solution of 2,4-dinitrochlorobenzene in 95 per cent alcohol. The beakers were allowed to stand for 1 hour or more at room temperature and then evaporated to dryness at 100–105° and heated 10 minutes between these limits of temperature in order to fuse the reagent with the substances to be determined. An excess of the dinitrochlorobenzene must be avoided, as it develops a yellow or orange color by itself when made alkaline. A small excess in the presence of 0.1 to 0.5 mg. of nicotinic acid or nicotinamide is permitted as the color is then too faint to affect the result significantly, but a blank must be run in order to make sure no significant color is produced by the reagent itself in the amounts added.<sup>5</sup> The 1 cc. of the reagent used by us is sufficient for developing maximum color in 0.1 to 0.5 mg. If more than this be taken, more reagent is needed. The heating should not be higher than 105°, as a brown color may develop at higher temperatures. The amide requires a longer heating to develop maximum color than does the acid. The amide should be heated for 10 minutes between 100–105°, while 5 minutes are enough for the acid. After this fusion, the fusion mixture is cooled to 25°, dissolved in 10 cc. of a clear solution of 0.1 per cent NaOH in 95 per cent alcohol which has been cooled to 10°, and filtered at once.

To test the accuracy of the method when the acid and amide

<sup>5</sup> Karrer and Keller avoided this difficulty by extracting the excess reagent from the melt with ether. This is an improvement on our method.

are mixed with the organic and inorganic matter of urine, urine was decolorized by filtering through charcoal and then treated with Lloyd's reagent and filtered. Known quantities of the amide or acid were then added to this urine and the amounts determined from the calibration curves made with the pure substances in water. Table I gives the results.

From these results, it is seen that when the procedure is carefully performed to prevent loss of material, and the reaction completed at temperatures consistent with minimum loss and maximum color development, the determination is accurate within 5.0 to 10 per cent. Errors in the determination of aqueous

TABLE I  
*Determination of Known Quantities of Nicotinic Acid and Amide*

Substance	Amounts determined (average of three or more readings)			
	1 cc.	2 cc.	3 cc.	5 cc.
	mg.	mg.	mg.	mg.
Nicotinic acid				
Solution in water, 5.5 mg. per 100 cc.	0.053	0.114		
Standard solution of acid in treated urine, 10 mg. per 100 cc.	0.109	0.197	0.31	0.509
Nicotinamide				
Solution in water, 3.6 mg. per 100 cc.	0.038	0.072		
Standard solution of nicotinamide in treated urine, 10 mg. per 100 cc.	0.094	0.21	0.293	0.48

solutions of the pure substances may be caused by incomplete reaction and possibly slight losses by vaporization. These, however, can be reduced to a minimum by care.

The next step was to add the known quantities of nicotinic acid or amide to fresh urine, before its decoloration, to see how accurately the amounts could be determined. Table II, containing the results, shows that both the acid and amide may be accurately determined within about 10 per cent when thus added. Furthermore, neither the acid nor the amide was absorbed by the charcoal used for decoloration of the urine. The per cent of error of Table II is calculated from the average of two or more determinations in each case.

Since the color is not permanent but slowly fades after filtration, the influence of the time factor was studied. The results (Table III) show that the maximum color slowly develops in the first 3 to 5 minutes and then fades, but in from 5 to 15 minutes the

TABLE II

*Determination of Nicotinic Acid and Nicotinamide Added to Untreated Urine*

Untreated urine + nicotinic acid	Conjugate present normally	Nicotinic acid added	Total present	Total determined	Error
cc.	mg.	mg.	mg.	mg.	per cent
1	0.016	0.106	0.122	0.12	-1.6
2	0.032	0.212	0.244	0.244	0.0
3	0.048	0.318	0.366	0.326	-10.9
Untreated urine + nicotinamide		Nicotinamide added			
1	0.016	0.057	0.073	0.079	+8.1
2	0.032	0.114	0.146	0.136	-8.2
3	0.048	0.171	0.219	0.224	+2.2

TABLE III

*Stability of Color at Successive Time Intervals*

Substance added	Per cent light transmission							
	30 sec.	1 min.	3 min.	4 min.	5 min.	10 min.	15 min.	20 min.
1 cc. nicotinic acid, 0.1 mg. per cc. water	86	82	81.2	80.3	81		82	82.5
1 cc. nicotinamide, 0.1 mg. per cc. water	76	72	70	67	65	67	70	
3 cc. nicotinic acid, 0.106 mg. per cc. urine		51	48	48		49		50
3 cc. nicotinamide, 0.057 mg. per cc. urine		53	49	48	48	49.1	51.2	52

per cent of transmission as read with the filter remains very constant.

*Method and Results When Applied to Urine*—In the urine of people who have ingested nicotinic acid or amide there might be trigonellin, nicotinic acid, nicotinic acid-glycine conjugate, and nicotinamide. It has been shown that in dog urine the acid,

the glycine conjugate, and trigonellin are present after ingestion of considerable quantities of nicotinic acid. In rabbit urine only the glycine conjugate and free acid are present (17, 18). Of these substances trigonellin, which is known to occur sometimes in human urine, gives no color reaction by our procedure. The acid, if present, gives a purple, the amide a red, and the color of the glycine conjugate has not been determined but probably would be a red. Actually we have found that if not more than 100 mg. of nicotinic acid are ingested, the color given by the urinary constituent is red as with the amide; but if large single doses of 100 to 500 mg. are ingested repeatedly, the color of the urinary constituent for the next few hours after each such dose is the purple color of nicotinic acid or its sodium salts. We interpret this to mean that in the first case either the amide or a conjugate is present. And only when large doses are given does the free acid or a salt appear in the urine.

The method worked out for the urine was as follows: 15 cc. of urine are decolorized by boiling with 0.1 to 0.3 gm. of Darco charcoal (Coleman and Bell). Crude vegetable charcoals do not absorb nicotinic acid (1). The solution when filtered should be clear and colorless. 3 cc. samples are then measured into 30 cc. beakers and are evaporated just to dryness in an oven at 80-100°. When dry, the beakers are removed and 1 cc. of an alcoholic solution of 2,4-dinitrochlorobenzene (1 gm. in 100 cc. of alcohol) is added. 10 mg. of 2,4-dinitrochlorobenzene are sufficient theoretically for development of maximum color with about 5 mg. of both acid and amide. After the samples have stood at room temperature with the reagent for 1 to 3 hours to allow for an intimate mixture, they are again evaporated to dryness and heated 10 minutes at 105°. The temperature must not exceed 105°. 10 minutes of heating at 105° gives the maximum color. Samples which are browned by overheating should be discarded.

The beakers are then cooled to 25°, or lower, and 10 cc. of a clear, cold (10°) solution of 0.1 per cent sodium hydroxide in 95 per cent alcohol are added and the residue stirred from the bottom. The colored solution is then made up just to 15 cc. by addition of about 5 cc. more of cold alcoholic sodium hydroxide. Since these alcoholic solutions are not clear they must be quickly filtered while cold. If for any reason the filtration is slow, it may be

facilitated by suction or centrifugation, but we have employed simply gravity, the solution running readily through the filter paper.

The quantitative reading must be made quickly (within 15 minutes) after the color has developed, although the chilled alcoholate retards the fading.

The colored but clear solution is transferred as soon as filtered to the photometer cell, and with a green filter the per cent of transmission of green light by the solution is read from the photometer scale.

A blank determination of the same quantities of the reagent with alkali alone should be run when fresh solutions are prepared, as too concentrated alkali produces a decided yellow color with any excess of the 2,4-dinitrochlorobenzene.

If the urinary color is a red like that caused by the amide, as it usually is, the amount is read from the amide curve and recorded as an equivalent amount of nicotinamide.

If it be a clear purple like that of nicotinic acid, the amount is read from the nicotinic acid curve.

If, however, as may happen after large doses of nicotinic acid have been ingested, the color is a mixture of red and purple, only an approximation or a semiquantitative estimation can at present be obtained with the photometer. This happens, however, only in specially treated patients or when nicotinic acid has been ingested for experimental purposes. Ordinarily the reading is from the amide curve. With the use of special filters the amount of each reacting substance might be estimated separately but with the three filters of the photometer at present available that is not possible.

### *Results*

No red, or purple, color-producing substances have been found to be present in the urine in all the cases of pellagrins in relapse or in the acute disease so far examined. None of these pellagrins has had enough nicotinic acid or its conjugates in the urine to develop color. We also found little or no color to develop in urine tests from normal persons who were maintained for some time on a strictly controlled "pellagra-producing" diet, a diet such as is usually eaten by pellagrins. The constituents of such a diet are

described later. However, the urine from a person suffering from inanition and dehydration, which came to our attention, showed a weak but positive reaction for nicotinamide, although her mouth was sore, like that of a pellagrin. The patient died before an accurate diagnosis could be made.

Several hundred urine specimens have been examined by this method both in normal persons after oral and intravenous administration of various amounts of nicotinic acid and its salts, and in disease. The urines from three pellagrins in relapse gave negative tests before treatment, but both they and persons on a pellagra-producing diet (*i.e.*, a limited diet without nicotinic acid-containing food) showed a quick response to nicotinic acid therapy by increased excretion of color-producing substances in the urine; but the excretion reverted to the low level normal for the individual on such a diet within 24 hours of ceasing ingestion of nicotinic acid.

The amount of nicotinic acid or its amide excreted daily by so called normal persons on usual diets has varied considerably, but the value as determined by the method on the urines of students, internes, and patients not ill of pellagra lay between 20 and 50 mg. daily.

After nicotinic acid was given by mouth, the quantity in the urine was much increased, but never was equal to the amount ingested. How much was converted into trigonellin has not been determined.

The attempt is being made to apply the reaction to blood filtrate, spinal fluid, gastric juice, and tissue hydrolysates. The results are not yet satisfactory, for although it is possible to see a deepening in the color developed in samples of the blood protein-free filtrate taken after nicotinic acid therapy as contrasted with the pretreatment blood, the color is masked by an intense yellow and is different from that of any of the pure substances so far examined.

We have also tested in a preliminary way the alcohol-water (70 per cent) extracts of foodstuffs, but only successfully when the extract was sufficiently light in color. A qualitative test on active liver concentrates, *i.e.* liver concentrates curative for pellagra and pernicious anemia, was positive, but not quantitatively satisfactory because of the natural color of the material.



In the case of dried brewers' yeast, hydrolyzed and unhydrolyzed samples gave small positive qualitative tests. Similar extractives of hydrolyzed and unhydrolyzed foodstuffs used as a pellagra-producing diet gave negative tests. Extractives of hominy grits, corn-meal, white flour, sweet potato, cabbage, spinach, pork fat, and sugar, foods constituting such a diet, all were negative. While these results are interesting as showing an important difference between the above foodstuffs and certain natural foods which contain pellagra-preventive substances, such as liver and yeast, the work is incomplete and the methods must be improved. Some of the disturbing pigments can no doubt be removed by proper solvents.

#### DISCUSSION

Until the present work was carried out there have been only two pyridine compounds known to occur in normal urine, namely, trigonellin and methyl pyridinium hydroxide. These substances already have the valences of the nitrogen saturated and this seems to prevent any addition product, such as is necessary to the first step of this reaction. For this reason, and because trigonellin has been found on trial not to give the reaction, we believe that neither of these substances will develop color under the conditions of this reaction, although we have not tested the N-methyl pyridinium hydroxide. Hence, a positive test, such as we have obtained, is fairly certain evidence of the presence of nicotinic acid, nicotinamide, certain of its salts, or, possibly, of its conjugated derivative, in normal human urine. Thus a third pyridine compound, nicotinic acid, is indicated as a constituent of normal human urine.

We conclude, after the examination of many specimens, that a failure of the urine to yield a positive reaction by this test indicates a serious deficiency of nicotinic acid or amide in the individual's diet. Hence this may prove to be a diagnostic sign of a prepellagrous condition, of value in those early cases in which the skin and mouth lesions are not yet well developed.

#### SUMMARY

1. A color reaction has been described for the detection of nicotinic acid, nicotinamide, substituted amides of nicotinic acid, and certain salts of the acid in urine and other fluids.

2. Colors are developed with nicotinic acid, nicotinamide, sodium nicotinate, and diethyl nicotinamide (coramine); no color is developed by trigonellin and picolinic acid.

3. By the application of this method to human urine it has been found that individuals on a normal diversified diet excrete daily color-producing substances equivalent to 20 to 50 mg. of nicotinic acid or its conjugates. We believe these substances to be nicotinic acid conjugates.

4. Pellagrins in relapse, or in the first acute disease, or normal individuals on a diet such as pellagrins usually consume, excrete little if any color-producing nicotinic acid derivatives.

5. Extracts of various foods, such as liver, yeast, and some others known to be pellagra-preventive foods, are shown to contain substances giving this nicotinic acid reaction, but the method does not yet permit a quantitative determination owing to the presence of other disturbing pigments.

6. The findings suggest that this method is useful as a confirmatory test of the pellagrous and prepellagrous states and for studying the excretion of nicotinic acid and other closely related substances which give colored products *in vitro* with 2,4-dinitrochlorobenzene, when alkalized.

7. Since nicotinic acid or the amide appears to be one of the vitamins, the method enables one to estimate the amount of this vitamin in urine.

8. Work is being continued on the estimation of the quantity of nicotinic acid and amide in different tissues and foods.

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## STEROL METABOLISM IN YOUNG WHITE RATS

### II. THE EFFECT OF SAPONIFIABLE LIPIDS AND DEGREE OF UNSATURATION OF LIPIDS ON THE STEROL METABOLISM OF THE WHITE RAT

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In a previous communication (1) it was shown that an increase in the fat content of the diet led to increased sterol content of the livers of young white rats. It was also demonstrated that larger negative sterol balances were obtained when the fat of the ration was increased. It was, therefore, believed that a greater sterol synthesis resulted from the increase in dietary fat. Rittenberg and Schoenheimer (2), who made use of deuterium in studying sterol synthesis in mice, state, "We interpret our results as ruling out a process by which cholesterol in the animal is formed by cyclization of fatty acids, and suggest instead that it is formed by the coupling of smaller molecules, possibly those which have been postulated to be intermediates in the fat and carbohydrate metabolism." They suggest that extra sterols were formed in the experiments of Eckstein and Treadwell (1) because more cholesterol was needed for the transportation of the larger amounts of fatty acids absorbed by the rats on the high fat diets. The writer is, at present, not concerned with the mechanism whereby sterols are formed from fat, but is undertaking to determine to what extent this takes place under different conditions. Schoenheimer and Breusch (3) concluded that no significantly larger amounts of sterols were synthesized by mice fed lard than when bread alone was fed. An examination of their data, however, shows that the animals fed lard actually synthesized 15 per cent more sterols than their controls on the bread diet. This percentage is lower than that which was found by Eckstein and Treadwell

(1), who fed soy bean and corn oils. The fats fed by these latter authors have higher iodine numbers and contain more non-saponifiable matter than lard, and it is, therefore, possible that the larger synthesis observed by them was due to these factors. In this connection, Minovici (4) reported that the sterol content of the incubated livers of dogs was greater in those animals which had previously ingested unsaturated fatty acids than in those fed saturated acids. He concluded that the degree of saturation of dietary fats plays a rôle in sterol synthesis.

It is thus evident that more information concerning the relation between the degree of saturation of dietary lipids and sterol formation is desirable. Data on the relationship between the saponifiable matter of a fat and sterol synthesis are also needed. With this in mind, the following experimental procedure was devised.

#### EXPERIMENTAL

Litters of young white rats (approximately 60 gm.) were divided into two groups and fed the diets shown in Table I for approximately 7 weeks. Records of food intakes were kept, feces collected, and changes in weight recorded. The sterols in the livers, remaining tissues, and feces were determined gravimetrically by the digitonin method, as previously outlined (1). The non-saponifiable matter and sterols in the diets and their accessories were also determined. An analysis of Diet 1D showed that it contained 12.8 gm. of fat (total ether-soluble extract), 14 gm. of protein ( $N \times 6.25$ ), 62 mg. of sterols, and 192 mg. of non-saponifiable matter per 100 gm. of food, as compared with 32 gm. of fat, 20.8 gm. of protein, 38 mg. of sterols, and 180 mg. of non-saponifiable matter in 100 gm. of Diet 2D. Thus, although Diet 2D contained almost 3 times as much fat as Diet 1D, it still had somewhat smaller amounts of sterols and non-saponifiable matter.

In view of its larger caloric content, it was to be expected that smaller amounts of Diet 2D would be ingested. Hence, the protein content of that diet was increased in order that the animals on this ration would ingest as much protein as their litter mates on Diet 1D. The food consumption of each rat is given in Table II. This summary shows that the animals on Diet 2D ingested almost twice as much fat as the other group. In con-

TABLE I  
*Composition of Diets*

All diets contained 4 per cent salt mixture\* and 2 per cent agar, and were supplemented with dry yeast tablets (400 mg.), viosterol, and corn oil containing 0.05 per cent carotene.†

Diet	Soy bean meal	Casein	Starch	Soy bean oil	Triolein	Corn oil	Coconut oil
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1D	35		54	5			
2D	49		25		20		
G		18	48				28
H		18	48			28	

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

† 2 drops of each of the fat-soluble vitamin concentrates were given every other day. The writer wishes to express his thanks to A. F. O. Germann of the S. M. A. Corporation for supplying the carotene used in this investigation.

TABLE II  
*Dietary Constituents Ingested by Individual Rats during Entire Experimental Period of Approximately 7 Weeks*

Diet No.	Rat No.	Soy bean meal	Fat	Protein	Sterols	Non-saponifiable matter
		gm.	gm.	gm.	gm.	gm.
1D	1	117	43	50	0.338	0.913
	2	195	71	83	0.479	1.331
	3	146	53	62	0.394	1.073
	4	176	64	75	0.451	1.227
	5	172	61	74	0.447	1.215
	6	148	54	64	0.381	1.084
Average.....		160	58	68	0.415	1.140
2D	7	164	106	69	0.269	0.873
	8	157	102	67	0.240	0.849
	9	178	117	76	0.259	0.925
	10	175	114	74	0.259	0.915
	11	160	103	67	0.267	0.859
	12	168	108	71	0.271	0.889
Average.....		164	108	70	0.261	0.885

trast with this, those on Diet 1D ingested approximately 60 per cent more sterols and 30 per cent more non-saponifiable matter than those on Diet 2D. The attempt to keep the protein and soy bean meal intakes of the animals constant was not entirely successful, but Table II shows that the differences in the intake between the groups of rats were not marked. The effects pro-

TABLE III

*Sterol Balances and Sterol Content of Livers, Remaining Tissues, Food, and Feces*

The balances were negative in each case.

Diet No.	Rat No.	Duration of experiment	Sterols					
			Liver	Remain- ing tissues	Feces (a)	Food (b)	(a - b)	Balance per day
		days	per cent	per cent	mg.	mg.	mg.	mg.
1D	1	51	0.38	0.23	685	338	347	6.8
	2	52	0.49	0.21	838	479	359	6.9
	3	53	0.27	0.23	697	394	303	5.9
	4	54	0.28	0.17	753	451	302	5.6
	5	55	0.31	0.19	764	447	317	5.8
	6	49	0.29	0.21	691	381	310	6.3
Average. ....			0.34	0.21	738	415	323	6.5
2D	7	57	0.57	0.23	1217	269	948	16.6
	8	47	0.47	0.21	575	240	334	7.1
	9	48	0.48	0.23	1064	259	805	16.8
	10	49	0.49	0.17	1031	259	772	15.7
	11	50	0.50	0.22	972	267	705	14.1
	12	49	0.49	0.21	1018	271	747	15.2
Average. ....			0.46	0.21	979	261	718	14.2

duced by these diets are shown in Table III. The gains in weight are not included, because they were all good and there were no essential differences in this respect between the two groups. Table III clearly shows that the greater negative balances occurred when Diet 2D was fed. These larger balances cannot be due to an augmented transfer of sterols from the tissues into the gut, because the livers of the rats on Diet 2D contained more

cholesterol than was present in the livers of their litter mates on Diet 1D, while the sterol content of the remaining tissues was the same for both groups.

It is logical to conclude that larger amounts of sterols were synthesized when larger amounts of saponifiable lipids were ingested. The fatty acids themselves thus appear to play a rôle in sterol synthesis. Whether this takes place directly or indirectly cannot be ascertained from these experiments. The writer does not desire to draw any conclusions concerning the effect that the non-saponifiable matter of a fat may have on the synthesis. While it may appear from the data obtained that this fraction plays no rôle because more sterols were synthesized on the diet containing the smaller amounts of non-saponifiable matter, it is, nevertheless, possible that the effect produced by this fraction of the lipids might have been overshadowed by the effect of the saponifiable lipids.

Diets G and H, described in Table I, were fed in a study of the influence of the degree of saturation on sterol balance. These diets differ from each other only with respect to fat. The iodine number (Rosenmund and Kuhnhehn (5)) of the corn oil was 118, as compared with a value of 8 for the coconut oil. Corn oil thus contains considerably more unsaturated fatty acids than coconut oil. The latter, on the other hand, contains large amounts of fatty acids of low molecular weight. Since the fat contents were the same for both diets, any differences observed, as a result of feeding these rations, must necessarily be due to the differences in the composition of the dietary fats.

Table IV shows that the average value for the liver sterols of the animals on the corn oil (Diet H) was 0.62 per cent as compared with 0.25 per cent for those on the coconut oil ration (Diet G). The averages for the negative daily sterol balances were 7.5 and 3.3 mg. respectively. It is evident, therefore, that larger amounts of sterols were present in the livers and greater negative balances were obtained when the more highly unsaturated fat was fed. As has been mentioned above, coconut oil contains large amounts of fatty acids of low molecular weight. Such acids have previously been shown (6, 7) to have a metabolism different from that of acids of high molecular weight, since the



TABLE IV

*Effect of Degree of Saturation of Dietary Lipids on Sterol Balances and Sterol Content of Livers and Remaining Tissues of Rats*

Rats 1 to 11 were from one litter, and Rats 21 to 29 were from another.  
All balances were negative.

Rat No.	Diet	Duration of experi- ment	Sterols					
			Liver	Remain- ing tissues	Feces (a)	Food (b)	(a - b)	Balance per day
		days	per cent	per cent	mg.	mg.	mg.	mg.
1	G	62	0.27	0.23	510	327	183	3.0
2		54	0.26	0.21	412	275	137	2.5
3		55	0.23	0.22	407	272	135	2.4
4		48	0.28	0.24	385	226	159	3.3
5		49	0.24	0.23	368	236	132	2.7
6		62	0.26	0.21	461	323	138	2.2
Average. ....			0.26	0.22	424	276	148	2.7
7	H	48	0.82	0.36	608	249	359	7.4
8		61	0.69	0.34	610	384	226	3.7
9		49	0.92	0.23	518	309	209	4.2
10		54	0.32	0.18	1312	353	959	17.7
11		61	0.50	0.27	638	346	292	4.8
Average. ....			0.69	0.26	737	328	409	7.5
21	G	46	0.15	0.21	474	291	183	4.0
22		52	0.26	0.20	662	330	332	6.4
23		58	0.24	0.22	480	359	121	2.1
24		50	0.28	0.23	534	321	213	4.3
Average. ....			0.23	0.22	538	325	213	4.2
25	H	46	0.38	0.21	656	374	282	6.1
26		48	0.68	0.23	721	373	348	7.2
27		57	0.33	0.20	936	467	469	8.2
28		53	0.43	0.20	832	427	405	7.6
29		49	0.77	Lost	796	392	404	8.2
Average. ....			0.52	0.21	788	406	382	7.5
" of all rats on								
Diet G. ....			0.25	0.22	470	296	174	3.3
Average of all rats on								
Diet H. ....			0.62	0.24	763	368	395	7.5

former are not deposited in the tissues. It seems unlikely, from this investigation, that the acids of low molecular weight are converted, to any appreciable extent, into cholesterol.

It follows that the nature of a dietary fat is an important factor in sterol synthesis by the white rat. These experiments, in conjunction with those summarized in Table III and those previously published (1), show that the quantity of fat and the chemical nature of the fat are important factors in sterol synthesis.

#### SUMMARY

1. The tissue content and balance of sterols have been determined in young white rats fed diets essentially the same in their content of preformed sterols and non-saponifiable matter, but different in their fat content (32 and 12.8 per cent respectively). The sterol synthesis was greater on the diet high in fat.

2. Young white rats fed diets containing 28 per cent fat synthesized larger amounts of sterols when the dietary fat was corn oil (iodine number, 118) than when coconut oil (iodine number, 8) was fed.

3. The quantity, as well as the chemical nature of the lipids in the diet, plays an important rôle in sterol synthesis by the white rat.

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## STEROL METABOLISM IN YOUNG WHITE RATS

### III. THE EFFECT OF HIGH AND LOW FAT DIETS ON THE STEROL BALANCES AND STEROL CONTENT OF THE HAIR OF YOUNG WHITE RATS

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The saponifiable lipids of the diet appear to play an important rôle in the production of sterols in the young white rat (1, 2). A greater synthesis of sterols was observed when the fat content of the diet was increased as well as when more highly unsaturated fats were fed. The average length of the experimental periods was 7 weeks. In preliminary experiments, data were obtained which indicated that the negative sterol balances decreased as the rats became older, and it was therefore desirable to carry out similar studies over long experimental periods. Since the sterol content of the lipids of the hair of young white rats has been shown to be high (12 per cent) (3), the sterols in the hair of rats were also studied under more carefully controlled conditions. The experimental diets differed only in the relative proportions of fat and carbohydrate. Both diets contained 18 per cent casein, 4 per cent of the Osborne and Mendel salt mixture (4), and 2 per cent agar, but Diet K contained 28 per cent of corn oil and 48 per cent of starch, while Diet L contained 5 per cent of corn oil and 71 per cent of starch. The dietary supplements and conduct of the experiments were the same as in the previously reported work (1, 2). Young white rats (60 gm.) were fed the diets for approximately 30 weeks. The experiment was divided into four periods, each of which was of about 7 weeks duration. Two litters of rats were used, each litter being divided into two groups and fed the high and low fat diets respectively. The feces collected during each period were analyzed separately.

At the end of the experiment, the hair of the rats was removed by shaving with a dry razor. It was weighed and hydrolyzed with 7.5 per cent alcoholic sodium hydroxide for 4 hours. The mixture was acidified with sulfuric acid and extracted four times with petroleum ether (b.p. 30–60°). The combined ether extracts were dried over anhydrous sodium sulfate for 48 hours and filtered; the solvent was evaporated off and the residue was dried to constant weight *in vacuo* at room temperature. This residue, which is designated in Table II as "Fatty acids + non-saponifiable matter," was dissolved in 95 per cent alcohol and the sterols were determined gravimetrically (1). The data obtained are summarized in Tables I and II.

Table I shows the gains in weight and the negative sterol balances. In calculating the averages for each group of rats for the individual periods, as well as for the total duration of the experiment, the values for the pregnant rats were omitted. Table I clearly shows that the largest negative balances occurred on the high fat diet. The balances calculated in terms of mg. per day are best for these comparisons. The average values for the rats on the high fat diet were always higher than those for their litter mates on the low fat diet. Table I shows that there are exceptions to this, but these are certainly few in number. The last column in Table I, in which the balances for the entire experiments are given, shows that the negative balances of all the rats on the 28 per cent fat diet were much higher than any of those of the rats on the 5 per cent fat diet. The average balances for the animals on Diets K and L were 1860 mg. and 622 mg. respectively.

One might conclude, from these values, that the animals on the high fat diet synthesized 3 times the amount of sterols produced by those on the low fat régime. The actual amounts of sterols synthesized by the two groups of rats, however, cannot be calculated from the difference between the fecal and dietary sterols, because it is generally accepted that a certain amount of cholesterol is always being catabolized (5). The extent to which this catabolism took place in the rats cannot be ascertained from the data obtained in this investigation and hence no accurate statement regarding the actual amounts synthesized by the rats can be made. Relatively, however, the data support the view that considerably

TABLE I

*Gains in Weight and Sterol Balances of Rats Fed High and Low Fat Diets for Approximately 30 Weeks*

Each period was of approximately 7 weeks duration. The letter preceding the rat number indicates the litter. Rats R1 to R6 and S1 to S5 inclusive were on the high fat diet (Diet K); the remaining rats were on the low fat diet (Diet L). All balances were negative.

Rat No.	Sex	Period 1		Period 2		Period 3		Period 4		Balance for entire experiment	
		Gain	Balance per day	Gain	Balance per day	Gain	Balance per day	Gain	Balance per day	Total	Per day
		gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	mg.	mg.
R1	M.	88	15.2	61	13.4	32	9.5	24	10.7	2559	12.4
R2	F.	89	19.0	30	6.7	20	5.4	16	9.2	1993	9.5
R3	M.	96	8.2	57	10.0	30	11.3	17	10.2	1918	10.4
R4	F.	80	10.9	96	5.3	51	6.9	13	10.8	1789	8.4
R5	"	83	13.2	26	7.4	20	10.9	Died		1628	10.0
R6	"	68	6.0	21	7.1	16	3.5	12	5.5	1164	5.6
Average.....		84	12.1	48	8.3	28	8.2	20	9.2	1885	9.3
S1	M.	100	14.6	69	14.2	51	9.4	10	8.1	2348	11.5
S2	"	84	14.4	35	9.2	23	8.6	9	7.1	1988	9.6
S3	F.	93	9.8	42	9.5	24	5.0	16	4.7	1509	7.3
S4	"	98	13.8	47	Pregnant	6	5.0	5	11.6	1497	10.2
S5	"	85	19.9	46	13.0	27	9.3	Pregnant		2158	14.3
Average.....		92	14.5	48	11.5	26	7.5	10	8.0	1835	9.4
R7	F.	69	9.1	24	1.8	16	1.1	4	1.0	668	3.2
R8	"	76	6.5	16	2.2	11	2.0	1	0.5	646	2.9
R9	"	84	2.7	4	2.7	20	2.1	5	1.5	463	2.2
R10	"	78	3.0	17	1.7	13	1.1	1	0.5	345	1.6
R11	M.	72	8.7	20	1.8	3	0.6	10	0.4	540	2.5
Average.....		76	6.0	16	2.0	13	1.4	4	0.8	532	2.5
S6	M.	135	3.8	41	1.4	18	2.3	4	0.5	440	2.0
S7	"	121	1.9	16	1.2	40	0.9	3	1.5	294	1.4
S8	F.	128	6.3	61	4.5	45	3.6	8	3.2	876	4.1
S9	"	126	5.4	26	3.6	14	4.6	6	3.2	852	4.1
S10	M.	116	3.2	51	3.9	32	2.4	5	1.8	594	2.9
Average.....		125	4.1	39	3.7	30	2.8	3	2.0	711	2.9

more sterols are synthesized by the rats on the high fat diet. Further consideration of the data shows that the sterol balances appear to decrease with age. The average value for the group of

TABLE II  
*Distribution of Lipids in Hair of Rats*

Rat No.	Diet	Fatty acids + non-saponifiable matter	Sterols in fatty acids + non- saponifiable matter	Sterols in hair	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
R1	K	4.96	14.2	0.70	
R2		5.12	15.6	0.80	
R3		6.10	11.9	0.73	
R4		5.84	11.0	0.64	
R6		5.29	14.2	0.75	
Average.....		5.46	13.4	0.72	
S1	K	6.38	11.2	0.71	
S2		4.36	15.3	0.67	
S3		5.01	16.0	0.80	
S4		4.23	15.8	0.67	
S5		7.51	9.3	0.70	
Average.....		5.50	13.5	0.71	
R7	L	3.91	16.7	0.65	
R8		4.23	17.0	0.72	
R9		4.27	17.6	0.75	
R10		4.62	15.9	0.73	
R11		4.60	16.3	0.75	
Average.....		4.33	16.7	0.72	
S6	L	6.89	12.0	0.83	
S8		5.64	12.6	0.71	
S9		4.87	14.1	0.69	
S10		5.00	13.6	0.68	
Average.....		5.60	13.1	0.73	
" Diet K.....		5.48	13.5	0.72	
" " L.....		4.89	14.9	0.73	

Litter R, fed the high fat diet, was 12.1 mg. per day for Period 1, as compared with a value of 9.3 for the whole experiment. The litter mates of these rats fed the low fat diet had average daily balances of 6 and 2.5 mg. respectively for the first division and the

entire experiment. Similar differences are to be found in the other litter. In comparing the individual balances in Period 1 with those in the other three periods, one finds that the relationship between sterol balance and age was not uniform. However, the fact remains that there is a considerable tendency to a greater negative balance in the younger animal. There appears to be a correlation between these negative sterol balances and increment in body weight. Thus, in the case of the females, the increases in weight parallel the negative balances. The relation is not so clear in the males on this diet, nor is it for either sex on the low fat diet, although there is a tendency in that direction.

In studying Table I from the point of view of sex, it is apparent that, so far as these data are concerned, no conclusions can be drawn concerning this factor.

The data in Table II are concerned with the analyses of the hair. These data show that there is no relationship between the fat content of the diet and the lipids of the hair. The average of the sum of the fatty acids and non-saponifiable matter in the hair of the rats in Litter R on the high fat diet is 5.46 per cent, as compared with 4.33 per cent for their litter mates on Diet L. On the other hand, the average values of the other litter are 5.46 per cent and 5.60 per cent respectively. A casual inspection of the second column of Table II might suggest that the diets did play a rôle, so far as the sterols in the hair are concerned, since the average value for the rats of Litter R on the low fat diet is considerably higher than the average for those on the higher fat diet. A further study of Table II, however, shows that these high values are due to the fact that the sums of fatty acids and non-saponifiable matter in this group were low and when, as was done in the last column, the calculation is made on the basis of the amount of sterols in the hair itself, the apparent differences disappear. Thus, there is no relationship between the sterols in the hair and the diets fed. This is in marked contrast with the values for the balances in this investigation and those previously reported for rat livers (1, 2).

#### SUMMARY

1. The negative sterol balances (fecal minus dietary sterols) of young white rats were 9.4 mg. per day on a 28 per cent fat diet and only 2.7 mg. per day on a 5 per cent fat diet.



2. With few exceptions, the balances decreased with age on both diets. The average values during Period 1 (approximately 7 weeks) on the high and low fat diets were 13.3 mg. and 5 mg. per day respectively, as compared with the respective values of 8.6 mg. and 1.4 mg. per day during Period 4.

3. Sex was found not to be a factor in sterol balances under the conditions of the experiment.

4. The sterol content of the hair of rats was found to be independent of the diets fed.

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## SURFACE DENATURATION OF EGG ALBUMIN

### A REPLY

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Wu and Wang (1) have recently criticized a paper of ours (2) dealing with the surface denaturation of egg albumin. They conclude that we were wrong in stating (a) that the higher the protein concentration, the lower the rate of denaturation and (b) that the process of surface denaturation is separable into two steps; namely, denaturation and coagulation.

The statement that the higher the protein concentration the lower the rate of denaturation was based on the velocity constants of the reactions, these varying in this order. It is true that when the rate is based on the actual amounts denatured, the rate is approximately independent of the protein concentration. On this point we are in agreement with Wu and Wang.

In regard to the second criticism that the process of surface denaturation cannot be separated into two steps, we are unable to explain the lack of agreement between ourselves and the Chinese workers. As indicated in our paper, the reaction consists of at least two phases, *i.e.* denaturation and coagulation. In this connection, a positive result is of more value than a negative one. To be absolutely certain of our position, we prepared egg albumin by the method of Kekwick and Cannan (3) from fresh hen's eggs, and electrodyalyzed the product to a specific conductivity of  $1.93 \times 10^{-5}$  reciprocal ohms. The solution was water-clear and the protein was 97.8 per cent heat-denaturable. Two 20 cc. portions of a 0.495 per cent protein solution containing sufficient HCl to bring the pH to 3.05 and 2.55, respectively, were shaken for 1 hour at 25°. At the end of this time the solutions were centrifuged

and the precipitates washed with distilled water and transferred to weighing bottles and dried to a constant weight. The clear supernatant protein solutions were brought to the isoelectric point with the addition of dilute NaOH and the fairly copious flocculum, which represented the uncoagulated surface-denatured protein, centrifuged and washed with distilled water. The precipitates were transferred to weighing bottles and dried to a constant weight at 104°. Two controls were run, one at pH 3.05 and the other at 2.55. Conditions were exactly the same as described above except that the solutions were not shaken, but remained quiescent for 1 hour and 45 minutes, after which time they were brought to the isoelectric point and centrifuged, and the amount of precipitate determined by drying to a constant weight. The following results were obtained.

pH of denatured solution.....	3.05	2.55
% coagulated upon shaking.....	6.67	2.96
% denatured but uncoagulated.....	3.91	5.12
% of control denatured.....	0.00	1.08
% denatured by shaking but uncoagulated....	3.91	4.04
Uncoagulated surface-denatured in % of total denatured.....	37.0	51.8

As can be seen, 37.0 per cent of the denatured protein was uncoagulated at pH 3.05, while at pH 2.55, 51.8 per cent was uncoagulated. These results clearly demonstrate that under adequately controlled conditions surface denaturation can be separated into two steps: (1) denaturation and (2) coagulation. We shall, therefore, insist that if the proper technique is used, our conclusion in regard to the two phase character of surface denaturation can be demonstrated.

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# THE PHOTOMETRIC DETERMINATION OF CYSTINE, CYSTEINE, ASCORBIC ACID, AND RELATED COMPOUNDS WITH PHOSPHOTUNGSTIC ACID\*

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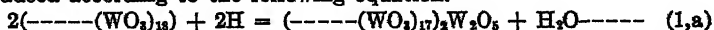
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Some time ago a brief report (1) was made on the use of the Pulfrich photometer for the determination of sulfhydryl and disulfide compounds by a modified Folin (2) method. Since then the method has been extensively used in this laboratory (3-9).

Folin's method is based upon the observation that cystine plus sulfite gives a blue color with phospho-18-tungstic acid; whereas cystine alone develops no color. The blue color is associated with the formation of phosphotungstic acid in which one of the 18 tungsten atoms is reduced (10), as indicated in Equation 1.<sup>1</sup> Clarke (12) and Lugg (13) have shown that in the action of sulfite upon cystine one-half is converted into S-cysteinesulfonic acid and the other half into cysteine (Equation 2). Cysteine is oxidized to cystine according to Equation 3, the phospho-18-tungstic acid (color reagent) acting as a hydrogen acceptor. In the presence of both sulfite and color reagent, the reactions of Equations 2 and 3

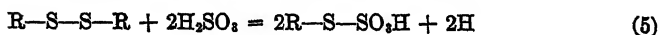
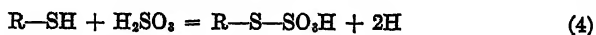
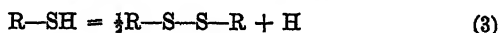
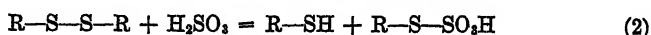
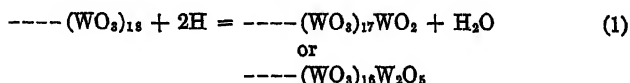
\* This report is from a dissertation submitted by Beatrice Kassel in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

<sup>1</sup> In a recent paper, Shinohara (11) claims that phospho-18-tungstic acid is reduced according to the following equation.



For the present paper it is of no consequence whether Equation 1 or 1,a is the correct one. All equations and values remain the same, since according to both equations two charges are transferred in the formation of 1 mole of reduced phosphotungstic acid.

proceed simultaneously, until all of the cysteine or cystine originally present is converted to cysteinesulfonic acid, as summarized in Equation 4 (Equation 2 + 2 × Equation 3) and in Equation 5 (2 × Equations 2 + 3).



Although many substances reduce the color reagent, it was assumed by Folin that the difference between the color obtained in the presence of sulfite and that obtained in the absence of sulfite could be calculated as cystine. After detailed investigations, Lugg (13) concluded that this procedure is unjustified, since the color developed by extraneous substances may be increased when sulfite is present and decreased when —SH compounds are reacting. He found it necessary, therefore, to estimate interfering substances by carrying out the reactions also in the presence of  $\text{HgCl}_2$ . Since  $\text{HgCl}_2$  prevents color development by —SH compounds, it is possible to estimate the contribution of extraneous reducers to the total coloration. Under Lugg's conditions, pH 5.7, cystine yields a maximum of color, while the color developed by interfering substances is considerably less than in more alkaline solutions. With the Pulfrich photometer, it was possible to devise a relatively simple and accurate method which retains the advantages of the Lugg modification.

*Pulfrich Photometer*—In the directions for the Pulfrich photometer (14),<sup>2,3</sup> the terminology used is different from that in the International Critical Tables (*cf.* (15)). We shall use only the latter.

<sup>2</sup> Directions for the use of the Pulfrich photometer, Mess 430 d/III (e) (1935), Carl Zeiss, Jena.

<sup>3</sup> Clinical colorimetry with the Pulfrich photometer, Mess 430 (f. e.) (1936), Carl Zeiss, Jena.

The Pulfrich photometer is calibrated in per cent transmission,  $D$  per cent  $= 100 I/I_0$ , and in density,  $d = \log I_0/I$ , where  $I_0$  is the intensity of the light entering the cell and  $I$  the intensity remaining after its passage through the cell. The extinction coefficient,  $K$ , is obtained from the density<sup>4</sup> by dividing by the cell length,  $l$  (in cm.).

$$K = d/l \quad (6)$$

The molecular extinction coefficient,  $\epsilon$ , is defined by the equation

$$\epsilon = K/c \quad (7)$$

where  $c$  is the molar concentration of the absorbing substance; that of reduced phospho-18-tungstic acid is related to the molar concentration ( $c_x$ ) of the substances which are responsible for the reduction

$$\epsilon = K/c_x \quad (8)$$

when 2 hydrogens are transferred,

$$\epsilon = 2K/c_x \quad (9)$$

when 1 hydrogen is transferred. The extinction coefficient for 1 mg. of reducing substance is given by

$$K \text{ per mg.} = \frac{\epsilon}{2M_x \times V} \quad (10)$$

where  $M_x$  is the *equivalent* weight of the reducing substance and  $V$  is the volume in cc. in which the determination is carried out.

#### Reagents—

1. Buffer mixture,<sup>5</sup> prepared by dissolving 105 gm. of citric acid ( $C_6H_8O_7 \cdot H_2O$ ) and 52.5 gm. of NaOH in about 450 cc. of water with cooling, then adding 110 gm. of solid sodium acetate ( $C_2H_3O_2Na \cdot 3H_2O$ ) and 250 gm. of solid urea. Separately prepared solutions of 13.6 gm. of  $ZnCl_2$  in 20 cc. of water and

<sup>4</sup> The older instruments are calibrated only in  $D$  per cent, so that the density has to be obtained either by calculation or from a table.

<sup>5</sup> Urea is incorporated in the buffer mixture to prevent turbidity (*cf.* (16)) and sodium acetate to neutralize the color reagent. The purpose of  $ZnCl_2$  and  $NH_4Cl$  has been discussed by Lugg (13).

26.8 gm. of  $\text{NH}_4\text{Cl}$  in 100 cc. of water are added; the mixture is diluted to 1 liter and preserved with toluene. It should be filtered before use. The pH of a mixture of 5 cc. of Reagent 1 with 1 cc. of color reagent is 5.7.

2. Sodium acetate solution (4 M). 544 gm. of  $\text{C}_2\text{H}_3\text{O}_2\text{Na} \cdot 3\text{H}_2\text{O}$  per liter.

3. Mercuric chloride (0.1 M). 2.72 gm. of  $\text{HgCl}_2$  per 100 cc.

4. Color reagent, prepared according to Folin's last communication (17) (molybdenum-free sodium tungstate can be obtained from the Mallinckrodt Chemical Works).

5. Buffered sodium sulfite, made by dissolving 9.5 gm. of  $\text{Na}_2\text{S}_2\text{O}_5$  in water, adding 15 cc. of 4 M sodium acetate, and diluting to 100 cc. This solution should be kept in the refrigerator and prepared fresh weekly.

6. Sodium hydroxide, 0.5 to 2 N.

7. Cadmium chloride (1 M). 22.8 gm. of  $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$  and 25 gm. of  $\text{NH}_4\text{Cl}$  per 100 cc.

*Development of Color*—The details of the preparation of Solutions A to E are given in Table I. Only Solutions A and C are prepared in duplicate. With urine and protein hydrolysates Solution E is not necessary; it is needed only if the unknown solution is strongly colored.

For pure solutions of S—S or —SH compounds it is necessary to prepare only Solution A or C respectively.

In carrying out the determination, 50 cc. glass-stoppered graduated cylinders are placed empty in a water bath at  $25^\circ \pm 1^\circ$ . The reagents given in Step I (Table I) are measured into the graduates. Step II, i.e., the addition of the unknown followed by NaOH, should not require more than 2 to 3 minutes for all solutions.<sup>6</sup> Step III is carried out separately for the individual graduates; the sulfite should follow the color reagent promptly or low results may be obtained (this was also observed by Shinohara (18)). The readings in the photometer should be completed within 15 minutes after the dilution of the solutions.

Color development can be conveniently carried out on a smaller

<sup>6</sup> If the unknown solution contains appreciable amounts of cysteine, Steps II to IV should be carried out separately for the individual solutions, care being taken that NaOH (Step II) is followed promptly by color reagent (Step III).

scale (*micromodification*). The procedure in Table I remains the same except that one-fifth of the amount of all reagents is accurately measured; the color is developed in 3 cc. and the final volume is 10 cc. Up to 1.6 cc. of unknown solution plus NaOH may be used.

*Reading of Solutions, Determination of  $K$ — $K(A)$ ,  $K(B)$ ,  $K(C)$ , and  $K(D)$* <sup>7</sup> ( $A$ ,  $B$ , etc., representing the solutions) are determined

TABLE I  
*Preparation of Solutions A to E*

Step in procedure	Amount of solution	Solution A	Solution B	Solution C	Solution D	Solution E
	cc.					
I	5	Buffer	Buffer	Buffer	Buffer	Buffer
	0.5		HgCl <sub>2</sub>		HgCl <sub>2</sub>	
	*	Water	Water	Water	Water	Water
II	Up to 8	Unknown	Unknown	Unknown	Unknown	Un- known
	†	NaOH	NaOH	NaOH	NaOH	NaOH
III	1	Color re- agent	Color re- agent	Color re- agent	Color re- agent	
	0.5	Sulfite	Sulfite			
IV	15	Invert and let stand 8 min. at 25°				
V	Dilute to 50 cc. with water, and complete readings within 15 min. in photometer					

\* Water should be added in such amounts that the final volume (including unknown solution and reagents not yet added) will equal 15 cc.

† A predetermined amount of NaOH (0.5 to 2 N) sufficient to bring the unknown solution to pH 5.7 is added separately.

with Filter<sup>8</sup> S-72 (Zeiss), with water or Solution E as compensating liquid. If  $K$  is very small (Solution D, and frequently also Solutions B and C), we find it advisable to make the reading with the measuring drum on the side of the unknown solution set at 50 per cent transmission.<sup>9</sup>

<sup>7</sup> Occasionally, Solution D becomes cloudy and should be filtered before being read.

<sup>8</sup> Clarke (12) has used a wave-length of 668 m $\mu$  for measuring the absorption by reduced phospho-18-tungstic acid.

<sup>9</sup> Directions for the use of the Pulfrich photometer, Mess 430 d/III (e), 14 (1935), Carl Zeiss, Jena.



Solutions A and B contain sulfite, which develops a small amount of color. In detailed experiments it was found that the sulfite blank ( $K$  (sulfite) = 0.01) is constant in Solution A although slightly variable in Solution B. It is sufficiently accurate to deduct 0.01 from the value of  $K$  found with Solutions A and B, so that  $K(A)$  and  $K(B)$  always include this correction.

*Standardization*—It can be seen from Table II that the same results were obtained with a number of specially purified substances<sup>10</sup> which give complete color development under our conditions (Table I). It is probable, therefore, that the molecular extinction coefficient of reduced phospho-18-tungstic acid is 8200 within the limits of error.<sup>11</sup> The same value for  $\epsilon$  was also obtained with a number of slowly reacting compounds under the conditions reported in the following paper. Different batches of molybdenum-free color reagent (17) have given the same results over a period of years (1).

Beer's law holds over a wider range of concentration than given in Table II, but the sulfite blank becomes an appreciable part of the total color with very small amounts of cystine. For less than 0.2 mg. of cystine it is preferable to use the micromodification.

$K$  per mg. of any reducing substance is found from Equation 10, with 8200 as the value of  $\epsilon$ ; thus  $K$  per mg. of cystine = 0.683 in the regular determination and 3.42 in the micromodification.

*Determination in Absence of Interfering Substances*—S—S compounds are measured by  $K(A)$  and —SH compounds by  $K(C)$ . If the solution contains only cystine,  $K$  (cystine) =  $K(A)$ ; if only cysteine is present,  $K$  (cysteine) =  $K(C)$  and  $K(A) = 2K(C)$ . If cystine and cysteine are present together,  $K$ (cysteine) =  $K(C)$  and  $K$ (cystine) =  $K(A) - 2K(C)$ . The content of the unknown in mg. of reducing substance is obtained by dividing by the value of  $K$  per mg.; e.g.,  $K$ (cystine)/0.683 = mg. of cystine in sample.

*Determination in Presence of Interfering Substances*<sup>12</sup> (*Urine and*

<sup>10</sup> We are indebted to Dr. V. du Vigneaud for the cystinyldiglycine.

<sup>11</sup> This is essentially the same as the value reported by us in 1935 (1). Recently Schöberl and Ludwig (19) described conditions for the Folin determination with the Pulfrich photometer which permit the estimation of certain S—S and —SH compounds in pure solutions. With cystine they obtained values for  $\epsilon$  ranging from 5300 to 8100 with concentrations from  $10^{-5}$  to  $4 \times 10^{-4}$  molar.

<sup>12</sup> Except ascorbic acid, which is discussed separately.

TABLE II  
*Standardization*

The extinction coefficients ( $K$ ) and molecular extinction coefficients ( $\epsilon$ ) are obtained with various compounds, which show complete color development in 8 minutes at 25°.

Substance	Solution	Amount	Concentration $c_x$	$K$	$\epsilon$
Substances reacting according to Equation 8, $\epsilon = K/c_x$					
Cystine	A	mg. per 50 cc.	$M \times 10^{-4}$		
		0.2	0.167	0.138	8270
		0.5	0.417	0.338	8120
		1.0	0.833	0.681	8170
		2.0	1.667	1.37	8240
Average.....					8200
Cystinyldiglycine Cysteine (+ sulfite)	A "	0.737	0.417	0.33 <sub>9</sub>	8130
		0.488	0.800	0.666	8260
		0.504	0.833	0.682	8190
Ascorbic acid	C	0.367	0.417	0.347	8320
		0.734	0.833	0.698	8380
		1.468	1.667	1.37	8230
Average.....					8310
Substances reacting according to Equation 9, $\epsilon = 2K/c_x$					
Fe <sup>++</sup>	D	0.093	0.333	0.143	8490
		0.232	0.832	0.349	8360
		0.343	1.23	0.491	8010
		0.465	1.67	0.708	8450
		0.929	3.33	1.37	8210
Average.....					8290
Cysteine	C	0.484	0.800	0.331	8280
		0.504	0.833	0.342	8210
Cysteinylglycine	"	0.741	0.833	0.34 <sub>9</sub>	8380
Thioglycolic acid	"	0.472	1.03	0.41 <sub>8</sub>	8150

*Protein Hydrolysates*)—Solution D contains HgCl<sub>2</sub> and no sulfite; no color is developed by cystine or cysteine. Interfering substances develop approximately the same amount of color in Solu-

tions C and D, so that  $K(\text{cystine}) = K(C) - K(D)$ . In the absence of cysteine,  $K(C) = K(D)$ .

Solution B contains  $\text{HgCl}_2$  and sulfite; no color is developed by cystine or cysteine. It was found that extraneous reducers do not develop the same amount of color in Solutions A, B, and D; this is in agreement with observations by Lugg (13). In Solution B, interfering substances develop more color than in Solution A, while in Solution D they develop less color than in Solution A.  $K(A) - K(B)$  therefore yields low results for  $K(\text{cystine})$ , while  $K(A) - K(D)$  yields high results. Detailed experiments indicated that approximately correct results are obtained by taking the average of the two blanks ( $K(B)$  and  $K(D)$ ), so that  $K(\text{cystine}) = K(A) - \frac{1}{2}(K(B) + K(D))$ . The error in the recovery of cystine in the presence of gelatin hydrolysate is very small (cf. Table III); experiments carried out with 0.2 to 1.0 mg. of cystine in the presence of 1 to 10 mg. of uric acid gave similar results.

If the determination is carried out in 50 cc. with 0.1 mg. of cystine or less, interfering substances develop approximately the same amount of color in Solutions A and B, so that  $K(\text{cystine})$  should be taken as  $K(A) - K(B)$  (cf. (13)).

When both cystine and cysteine are present,

$$K(\text{cystine}) = K(A) - \frac{K(B) + K(D)}{2} - 2K(C - D)$$

*Interference by Heavy Metals*—Heavy metals may affect the determination in various ways: by reducing the color reagent ( $\text{Cu}^+$ ,  $\text{Fe}^{++}$ ), by reacting with  $-\text{SH}$  compounds ( $\text{Hg}^{++}$ ,  $\text{Cd}^{++}$ ), by causing fading ( $\text{Cu}^{++}$ ), by reacting with the sulfite ( $\text{Sn}^{++++}$ ), etc. The effect of  $\text{Fe}^{++}$  and of traces of  $\text{Cu}^+$  and  $\text{Cu}^{++}$  is eliminated in our system of analysis. Other metals and larger amounts of copper should be removed from the unknown solution.

Should it be desirable to determine cystine after cuprous chloride precipitation ((20), cf. (6, 21, 22)), the photometric method can be used, the copper being removed either by  $\text{H}_2\text{S}$  (in this case cysteine is liberated) or by KCNS (cystine liberated). The Sullivan determination can be carried out on the KCNS filtrate after removal of the last traces of copper by pyridine.<sup>13</sup>

<sup>13</sup> Pyridine gives a precipitate with phospho-18-tungstic acid, as observed by Wu (10).

*Ascorbic Acid; Simultaneous Determination of Cystine, Cysteine, and Ascorbic Acid*—Ascorbic acid reduces phospho-18-tungstic acid mole for mole according to Equation 1. The data in Table II indicate that ascorbic acid in pure solution can be accurately determined over a wide range of concentration.

Ascorbic acid develops the same amount of color in Solution A as in Solution C. Solution D becomes cloudy ( $\text{HgCl}_2$ ), but does not develop any color (or only a trace), because most of the ascorbic acid is oxidized by the  $\text{HgCl}_2$  before the color reagent is added. In Solution B ascorbic acid is also oxidized by the  $\text{HgCl}_2$ , but on addition of the sulfite considerable color develops.<sup>14</sup>

It is not possible to determine cystine, cysteine, and ascorbic acid simultaneously, with the procedure in Table I. A special method was therefore devised, based on the observation that  $\text{CdCl}_2$  retards the oxidation of ascorbic acid by  $\text{HgCl}_2$ , but not by phospho-18-tungstic acid. S—S and —SH compounds, on the other hand, give no color in the presence of a mixture of  $\text{CdCl}_2$  and  $\text{HgCl}_2$ .

The determinations require five solutions: Solutions A and C (cf. Table I), and Solutions  $B_1$ ,  $D_1$ , and  $D_0$  described in Table IV.

Ascorbic acid develops the same amount of color in Solutions A,  $B_1$ , C, and  $D_1$ , but no color in Solution  $D_0$ . Cystine and cysteine develop no color in Solutions  $B_1$ ,  $D_1$ , and  $D_0$ . Other interfering substances develop approximately the same amount of color in Solution  $B_1$  as in Solution B and in Solutions  $D_1$  and  $D_0$  as in Solution D. The examples in Table V indicate how  $K(\text{cystine})$ ,  $K(\text{cysteine})$ , and  $K(\text{ascorbic acid})$  are calculated.

*Applications*—The method has been extensively used in the analysis of normal and cystinuric human urine (3–5) and also with normal and cystinuric dog urine (6).

Representative results with lactalbumin (Labco 7-HAAX<sup>15</sup>)

<sup>14</sup> Oxidation of ascorbic acid by  $\text{HgCl}_2$  is known to be reversible (23), so that some color is gradually developed in Solution B, which contains  $\text{HgCl}_2$  and sulfite. The oxidation of ascorbic acid by the color reagent is apparently irreversible, since sulfite has no effect (Solution A).

<sup>15</sup> Obtained through the courtesy of Dr. G. C. Supplee. Only one of the lactalbumin preparations (used for Experiments I to III in Table VI) contained some cysteine.

TABLE III  
*Cystine Determinations in Presence of Gelatin Hydrolysate*

Cystine	Hydrolyzed gelatin	$K \left( A - \frac{B+D}{2} \right)$	Cystine recovered	Error
<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	<i>mg.</i>
0.2	200	0.148	0.22	+0.02
	100	0.148	0.22	+0.02
	50	0.134	0.20	0
	20	0.133	0.19	-0.01
0.5	200	0.345	0.51	+0.01
	100	0.338	0.50	0
	50	0.342	0.50	0
	20	0.343	0.50	0
1.0	200	0.697	1.02	+0.02
	100	0.703	1.03	+0.03
	50	0.699	1.02	+0.02
	20	0.695	1.02	+0.02
2.0	200	1.33 <sub>7</sub>	1.96	-0.04
	100	1.35 <sub>8</sub>	1.98	-0.02
	50	1.38 <sub>8</sub>	2.02	+0.02
	20	1.39 <sub>8</sub>	2.03	+0.03

TABLE IV  
*Preparation of Solutions B<sub>1</sub>, D<sub>1</sub>, and D<sub>0</sub>*

Step in procedure	Amount of solution	Solution B <sub>1</sub>	Solution D <sub>1</sub>	Solution D <sub>0</sub>
	cc.			
I	5	Buffer	Buffer	Buffer
	1			HgCl <sub>2</sub>
	2	CdCl <sub>2</sub>	CdCl <sub>2</sub>	
II	*	Water	Water	Water
	Up to 6	Unknown	Unknown	Unknown
	†	NaOH	NaOH	NaOH
				Let stand for 5 min.
III	0.5	HgCl <sub>2</sub>	HgCl <sub>2</sub>	
	1	Color reagent‡	Color reagent‡	Color reagent
	0.5	Sulfite		
IV	15	Let stand 8 min. at 25°		
V	Dilute to 50 cc. with water, filter Solution D <sub>0</sub> , and complete readings within 15 min. in photometer			

\* Water should be added in such amounts that the final volume (including unknown solution and reagents not yet added) will equal 15 cc.

† A predetermined amount of NaOH sufficient to bring the unknown solution to pH 5.7.

‡ The color reagent must be added immediately after the HgCl<sub>2</sub> or part of the ascorbic acid will be oxidized by the HgCl<sub>2</sub>.

TABLE V  
Determination of Ascorbic Acid, Cystine, and Cysteine

Experiment No.	Substance	Amount	Extinction coefficients					
			K(A)	K(B)	K(B <sub>1</sub> )	K(C)	K(D <sub>0</sub> )	K(D <sub>1</sub> )
		mg.						
I	Cystine +	0.25	0.501			0.159		
	Cysteine	0.25						
II	Cystine +	0.5	0.677			0.172		
	Cysteine	0.25						
III	Cystine +	0.5	1.012			0.344		
	Cysteine	0.5						
IV	Cysteine +	0.504	0.974	+++	0.341	0.700	0	0.346
	Ascorbic acid	0.367						
V	Cystine +	0.25	0.847	+++	0.350	0.519	0	0.350
	Cysteine +	0.25						
	Ascorbic acid	0.367						
VI	Normal Urine B +	4 cc.	0.689	+++	0.322		0.018	0.343
	Cystine +	0.5						
	Ascorbic acid	0.367						
VII	Normal Urine B	4 cc.	0.081	0.035		0.018	0.018	

Calculations and Results

	Cysteine		Cystine		Ascorbic acid	
	K(C)	Recovery	$\frac{K(A) - 2K(C)}{2}$	Recovery	$\frac{K(D_1) - K(D_0)}{K(D_0)}$	Recovery
		mg.		mg.		mg.
I	0.159	0.23	0.183	0.27		
II	0.172	0.25	0.333	0.49		
III	0.344	0.50	0.324	0.47		
	$\frac{K(C) - K(D_1)}{K(D_1)}$		$\frac{K(A - \frac{B_1 + D_1}{2}) - 2K(C - D_1)}{2}$			
IV	0.354	0.52			0.346	0.37
V	0.169	0.25	0.159	0.23	0.350	0.38
VI			0.356	0.46*	0.325	0.35
	$\frac{K(C) - K(D_0)}{K(D_0)}$	Content	K(A - B)	Content	$\frac{K(C) - K(D_0)}{K(D_0)}$	Content
VII	0	0	0.046	0.067	0	0

\* Corrected for the cystine content of the urine (Experiment VII).

TABLE VI

*Analysis of Lactalbumin (Labco 7-HAAX) and Reduced Lactalbumin*HCl hydrolysis, 130° oil bath, 8 hours, under CO<sub>2</sub>; HI hydrolysis, 150° oil bath, 6 hours, under CO<sub>2</sub>.

Experiment No.	Hydrolysis			Method	Cystine	Cystine	Cystine + cystine
	Lactalbumin	Acid					
	mg.		cc.				
I	1380.2	6 N HCl	27.5	Photometric Folin-Marenzi Sullivan	2.67	0.39	3.06 3.37 3.08
II	475.3	6 " "	5	Photometric Sullivan	2.67	0.32	2.99 3.15
III	432	6 " "	10	Photometric Sullivan	2.72	0.29	3.01 3.13
IV	245.5	6.5 N HCl in 42% formic acid	5	Photometric Sullivan	3.18	0	3.18 2.90
V	22.04	6.5 N HCl	1	Microphoto- metric	3.09	0	3.09
VI	10.30	6.5 " "	1	"	2.98	0	2.98
VII	17.52	6.5 " "	0.5	"	3.06	0	3.06
VIII	495.6	57% HI	10	Baernstein*			3.08
IX	480.9	57% "	10	"			3.09
Average.....				Photometric Microphoto- metric Sullivan Baernstein*			3.06 3.04 3.06 3.09

Cystine content of lactalbumin ..... 3.1

		Reduced lact- albumin					
X.	Batch 14	233.5	6 N HCl	7	Photometric Sullivan	0.69	2.31
XI.	" 17	215.4	6 " "	7	Photometric Sullivan	0.53	2.14
XII.	" 17	304.7	57% HI	10	Baernstein*		
							3.00 4.30 2.67 4.00 3.23

\* All values with the Baernstein method are corrected for H<sub>2</sub>S formation (8).

and with reduced lactalbumin<sup>16</sup> are given in Table VI. With lactalbumin the same results were obtained by the photometric, microphotometric, Baernstein (24, 8), and Sullivan methods; the value by the Folin-Marenzi method was about 10 per cent high.

In the analysis of reduced lactalbumin, the Baernstein method indicated about the same amount of cystine plus cysteine as in the non-reduced protein, while the photometric method clearly showed that about 75 per cent of the original cystine was present as cysteine. The values obtained by the Sullivan method are much too high.<sup>17</sup> In the analysis of Batch 17 of reduced lactalbumin (Table VI), the cystine plus cysteine content found by the photometric method was 2.67 per cent and that found by the Baernstein method was 3.23 per cent. However, the discrepancy is probably not due to errors in the determinations, but is caused by a loss of cysteine due to humin formation during the hydrolysis with HCl. Lugg's experiments (25) indicate that in the hydrolysis of proteins with HCl the loss of cystine originally present is very small, but the loss of cysteine might be very serious. On the other hand, with HI hydrolysis (Baernstein method), there is no humin formation and the losses of both cystine and cysteine are usually negligible (except for slight H<sub>2</sub>S formation (cf. (8))). The values for cystine plus cysteine obtained by the Baernstein method and the value for cystine by the photometric method are therefore approximately correct. The value for cysteine found with the photometric method is probably too low, and the actual cysteine content of this preparation of reduced lactalbumin can be assumed to be about 2.5 per cent.

The cystine content of a number of other proteins has been determined by the photometric method and will be reported in a separate paper.

We are indebted to Dr. V. du Vigneaud for reading this manuscript and for valuable criticism.

<sup>16</sup> The protein was reduced with thioglycolic acid under N<sub>2</sub>. The details of the preparation will be described elsewhere.

<sup>17</sup> Theoretically, cysteine should give twice as much color as cystine in the Sullivan method, but only about 1½ times the amount of color is obtained.



## SUMMARY

1. A photometric modification of the Folin-Lugg method for the determination of cystine and cysteine is described; accurate results are obtained in the presence of extraneous reducers.

2. The photometric determination of ascorbic acid is described. A special method has been developed for the simultaneous determination of cystine, cysteine, and ascorbic acid.

3. A micromodification is described, which permits the determination of cystine and cysteine, with 10 to 20 mg. of protein for hydrolysis.

4. Representative results obtained with lactalbumin and reduced lactalbumin by the photometric, microphotometric, Folin-Marenzi, Baernstein, and Sullivan methods are given.

5. Certain aspects of the theory of spectrophotometric colorimetry are discussed.

6. The molecular extinction coefficient of reduced phospho-18-tungstic acid, determined with a number of reducing substances, yields the same value ( $\epsilon = 8200$  with Filter S-72) in every case.

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# THE RATE OF REACTION OF SULFHYDRYL AND DISULFIDE COMPOUNDS WITH PHOSPHO- TUNGSTIC ACID AND WITH SULFITE\*

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Some years ago, Brand, Harris, and Biloon (1) observed that in the Folin-Marenzi method equivalent amounts of cystine and cystine dimethyl ester dihydrochloride develop exactly the same amount of color, while dialanylcystine gives less color. In our earlier report (2), it was noted that in the photometric method, cystine homologues show a slower rate of color development than cystine itself and that the rate decreases with increasing molecular weight. In the present paper, the behavior of a number of sulfur compounds is described in greater detail; the reaction rates were studied and the influence of the sulfite concentration and of the addition of cystine or cysteine on these rates determined.

## EXPERIMENTAL

The experiments are reported in Table I and Figs. 1 to 10. For the pentocystine (3), hexocystine (4), and cystinyldiglycine (5) we are indebted to Dr. V. du Vigneaud, for the cystine ester to Dr. H. T. Clarke, for the cystamine to Dr. M. X. Sullivan, and for the ergothioneine to Dr. J. A. Behre; homocystine and  $\gamma, \gamma$ -dithiodibutyric acid were prepared as described previously (6, 7). The  $-\text{SH}$  compounds (except glutathione) were obtained by reduction of the corresponding  $\text{S}-\text{S}$  compounds with zinc in 2

\* This report is from a dissertation submitted by Beatrice Kassel in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

per cent HCl (8). 10 mg. of cystine (or an equivalent weight of the other S compounds) were dissolved in 20 cc. of 2 per cent HCl and Zn dust was added, 300 mg. at once and 100 mg. after  $\frac{1}{2}$  hour; the mixture was kept at room temperature for 1 hour and filtered. The resulting solutions of the —SH compounds were used immediately for iodometric and photometric analyses. The iodometric titration was repeated after 1 hour, and the results always indicated that no deterioration had taken place during that time.

The iodometric titrations were carried out, *in a stream of N<sub>2</sub>*, under the conditions established by Lavine (9). The results of these titrations indicated complete reduction, except for homocysteine with which only 86 per cent was accounted for as —SH compound. Homocysteine is known to form a lactone (10, 11) which cannot be titrated with iodine.

The photometric determinations were carried out with Solutions A and C (*cf.* (12) Table I) and with a new solution, A<sub>2</sub>, which is the same as Solution A, except that it contains 2 cc. of sulfite<sup>1</sup> ((12) Reagent 5). The buffer mixture used ((12) Reagent 1) did not contain urea. In the preparation of these solutions, the standing and dilution to 50 cc. ((12) Table I, Steps IV and V) were omitted, so that color development could continue over a long period of time in a volume of 15 cc. Immediately after completion of Step III,<sup>2</sup> the solutions were mixed and a portion transferred to an absorption cell of suitable length, the remainder being kept at  $25^{\circ} \pm 1^{\circ}$  in the stoppered graduates. The first reading was taken 2 minutes after the completion of Step III, and further readings were made after 4, 8, 15, 30, 45, 60, 120, and 180 minutes. The temperature of the solutions while they were in the absorption cells was not controlled, but experiments were carried out only when the room temperature in the neighborhood of the instrument was  $25^{\circ} \pm 2^{\circ}$ .

An outline of the experiments is given in Table I. In all experiments except I-Cye and II-Cye, the amounts of —SH and S—S

<sup>1</sup> With 2 cc. of sulfite,  $K(\text{sulfite}) = 0.025$ . This should be deducted from all  $K(A_2)$  values.

<sup>2</sup> The color reagent was always added immediately after the S—S or —SH compound. When cystine or cysteine was also added, the mixture of S compounds and buffer never stood for more than 30 seconds before the addition of the color reagent.

compounds were chosen so as to be equivalent to 0.5 mg. of cystine, *i.e.*  $4.17 \times 10^{-3}$  mm per 15 cc. for the —SH compounds and  $2.08 \times 10^{-3}$  mm for the S—S compounds. Experiments I-Cye and II-Cye were carried out with one-half the amount of —SH compounds, *i.e.*  $2.08 \times 10^{-3}$  mm per 15 cc. The amount of

TABLE I

*Outline of Experiments Reported in Figs. 1 to 10*

Color developed in 15 cc. at 25°.

Series No.	Fig. No.	Solution	Sulfite content	Amount		Complete color development*		
				Cysteine	—SH compound	—SH compound + cysteine	—SH compound	
				mm $\times 10^{-3}$ per 15 cc.	mm $\times 10^{-3}$ per 15 cc.	K	K	per cent
0	1	C	0	0	4.17		1.14	100
0-Cye	2	"	0	4.17	4.17	2.28	1.14	100
I	3	A	0.5	0	4.17		2.28	200
I-Cye	4	"	0.5	2.08	2.08	2.28	1.14	200
II	5	A <sub>2</sub>	2	0	4.17		2.28	200
II-Cye	6	"	2	2.08	2.08	2.28	1.14	200
				Cysteine	S—S compound	S—S compound + cysteine	S—S compound	
				mm $\times 10^{-3}$	mm $\times 10^{-3}$			
III	7	A	0.5	0	2.08		1.14	100
III-Cy	8	"	0.5	2.08	2.08	2.28	1.14	100
IV	9	A <sub>2</sub>	2	0	2.08		1.14	100
IV-Cy	10	"	2	2.08	2.08	2.28	1.14	100

\* K for complete color development is calculated from  $\epsilon = 8200$  (*cf.* (12)).

cystine or cysteine added was 0.5 mg. in all the experiments except I-Cye and II-Cye, in which it was 0.25 mg.

In Figs. 1 to 10 the extinction coefficients found in the various experiments are given in per cent of that (1.14) observed with cysteine (without sulfite). For instance, in an experiment with an S—S compound in Series III or IV, K equal to 0.57 is reported as 50 per cent. A value of K equal to 1.71 found in an experiment with an —SH compound in Series I or II is reported as 150 per cent. In the Cye and Cy series the values reported are corrected

for the color developed by cystine or cysteine. In the 2 and 4 minute experiments of Series I to IV, in which the color development by cystine and cysteine is incomplete, the corrections were made in accordance with the corresponding values obtained for cystine and cysteine. For example, in Fig. 8, with homocystine (Series III-Cy) at 2 minutes, the value for  $K$  found was 1.38. Color development for cystine (Series III) is 76 per cent ( $K = 0.87$ ); deducting this from 1.38 leaves a value of  $K = 0.51$  for homocystine, *i.e.* 45 per cent color development. The correction for color development by cystine or cysteine was thus made uniformly on the basis of the results given by cystine or cysteine alone.

The results are easily reproducible as far as complete color development is concerned, but the values for incomplete color development during the first few minutes are somewhat variable, mostly because the temperature could not be properly controlled ( $\pm 2^\circ$ ).

The presence of zinc has no influence upon the rate of color development as indicated by experiments with ergothioneine, glutathione,  $\gamma$ -thiobutyric acid, and with cystine, homocystine, and hexocystine.

*Series O.* —*SH Compounds without Sulfite (Fig. 1)*—*SH* compounds are oxidized to *S—S* compounds according to Equation 1, phospho-18-tungstic acid (color reagent) acting as a hydrogen acceptor.



Complete color development was obtained with a number of *—SH* compounds, and the value of  $K$  agreed within the experimental error with that calculated from the molecular extinction coefficient (*cf.* (12)).

The reaction is complete in 2 minutes with cysteine, cysteine ethyl ester, cysteinylglycine, and thioglycolic acid. Alanylcysteine and glutathione take 2 to 3 hours for complete color development. This is a measure of the reaction rate of these two compounds only if we assume that no hydrolysis takes place.

With homocystine, color development increases continuously, but is still incomplete (75 per cent) after 3 hours. The homo-

cysteine solution used in these experiments contained only about 14 per cent of lactone,<sup>3</sup> as found by the iodometric titration.

With hexocysteine, pentocysteine, and  $\gamma$ -thiobutyric acid, color development is incomplete, but practically the same value is obtained at 2 minutes as at 3 hours. The peculiar behavior of these

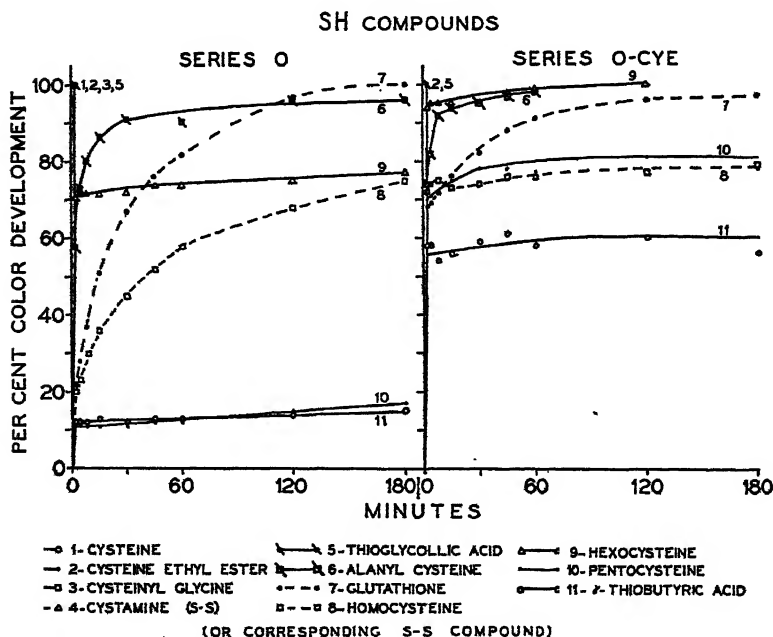


Fig. 1

Fig. 2

substances might possibly be due to ring formation, although the iodometric titrations accounted for all the sulfur as free —SH groups.<sup>4</sup> The results obtained with these three —SH compounds

<sup>3</sup> It is not likely that appreciable lactone formation occurred during the 3 hours of color development at pH 5.7, since Riegel and du Vigneaud (11) have shown that the rate of ring formation increases with the acid concentration.

<sup>4</sup> In other experiments with  $\gamma$ -thiobutyric acid, the iodometric titration indicated only about 90 per cent of free —SH groups. Thus there may be some ring formation but not enough to account for the very slight color development in Series O.

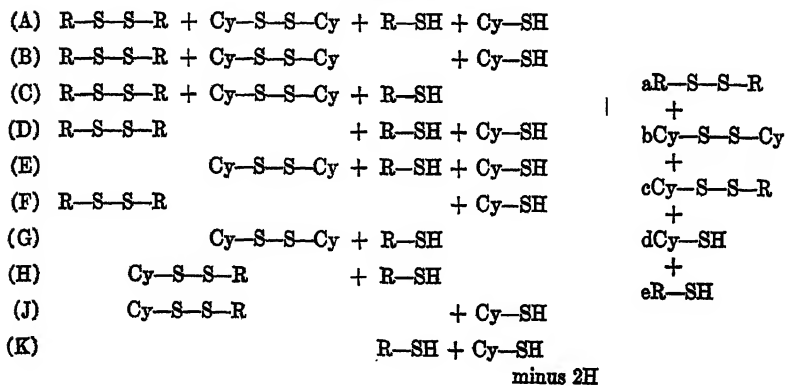


were not very consistent, the greatest variations being observed with hexocysteine. No attempt will be made to explain the peculiar behavior of these three substances.

Ergothioneine does not reduce phospho-18-tungstic acid under our conditions, *i.e.* at pH 5.7.

*Series O-Cye.* —SH compounds without Sulfite in Presence of Cysteine (Fig. 2)—If cysteine is present at the same time as a slowly reacting —SH compound, certain complications arise, owing to the interaction of unchanged R—SH with cystine, the latter being formed rapidly according to Equation 1. The various possibilities are presented in Equation 2, in which Cy—SH and Cy—S—S—Cy represent cysteine and cystine, and R—SH and R—S—S—R any other —SH and S—S compound.

*Equation 2*



The formation of mixed disulfides was also taken into account, since Toennies (13) recently prepared a mixed disulfide of cysteine and thiourea, and since a mixed disulfide of cysteine and thio-glycolic acid was demonstrated in the urine of normal and cystinuric human beings after the administration of carboxymethyl cysteine (14).

It can be seen from Fig. 2 that with the rapidly reacting —SH compounds, the addition of cysteine has no perceptible effect. With GSH plus cysteine, there is a marked acceleration of the reaction in the first 2 minutes, but total color development takes approximately the same time as without cysteine. The simplest explanation of this speeding up is on the basis of Reaction G

(Equation 2), the increased color during the first 2 minutes being due to formation of additional amounts of cysteine. With alanyl-cysteine plus cysteine, there is also considerable speeding up, especially in the first 2 minutes; complete color development is attained faster than without cysteine.

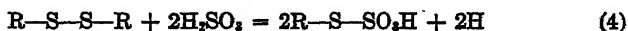
With the other —SH compounds, the reaction is likewise speeded up during the first 2 minutes, but then comes practically to a standstill. Hexocysteine shows complete color development in about 1 hour. With homocysteine, color development after 3 hours was about the same as without cysteine. The speeding up with homocysteine may be explained as discussed under GSH; the incomplete color development is probably due to the presence of lactone.

With pentocysteine and  $\gamma$ -thiobutyric acid, the rate of reaction is markedly increased during the first 2 minutes, but no further reaction takes place during the next 3 hours. The peculiar behavior of these two compounds remains unexplained, particularly in view of the finding that complete color development can be obtained with sulfite (*cf.* Series I and II).

*Series III and IV.* S—S Compounds with Sulfite (Figs. 7 and 9)—S—S compounds react with sulfite according to Equation 3.



In the presence of a hydrogen acceptor (color reagent), the reactions of Equations 1 and 3 proceed simultaneously until all of the S—S compound is converted into the sulfonic acid, as summarized in Equation 4.



The rates of reaction of Equations 1 and 4 can be found experimentally, but that of Equation 3 can be inferred only if the reactions of Equations 1 and 3 proceed independently of each other and if the reaction of Equation 3 is much slower than that of Equation 1. This has been realized with certain compounds.

The rate of splitting of S—S compounds by sulfite (Equation 3) is dependent on the sulfite concentration. With all the compounds tested color development is considerably faster with 2 cc. of sulfite (Fig. 9), and complete color development is reached within 1 hour, yielding the same value for  $\epsilon$ .

# SH COMPOUNDS

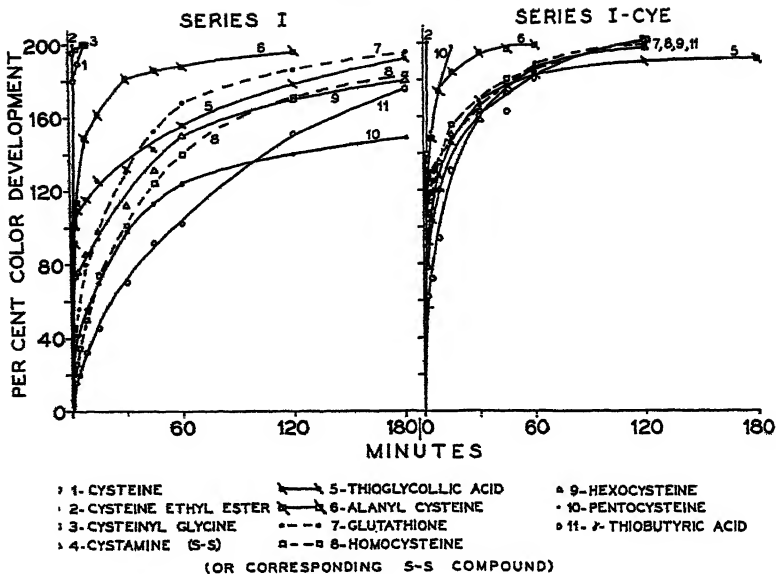


Fig. 3

Fig. 4

# SH COMPOUNDS

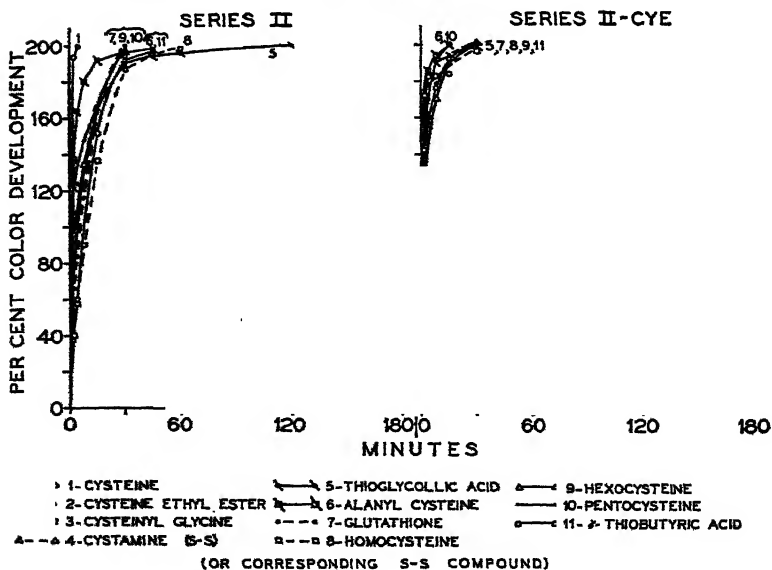


Fig. 5

Fig. 6

From Fig. 7 it can be seen that with cystine ester, cystinyl-diglycine, cystine, dithiodiglycolic acid, and dialanycystine the reaction of Equation 4 is slower than that of Equation 1, which is practically instantaneous (except with alanyl-cysteine, *cf.* Series 0), so that the rate of color development indicates the rate at which these compounds react with sulfite. The cystine derivatives substituted in the carboxyl group are split faster by sulfite than cystine, while dithiodiglycolic acid reacts particularly slowly. Cystamine reacts more slowly than cystine, but the corresponding —SH compound was not investigated.

An interpretation of the reaction rates of homo-, pento-, and hexocystine, and dithiodibutyric acid cannot be attempted, since the reaction of the corresponding —SH compounds (Equation 1) is complicated either by ring formation or by other factors (*cf.* Series 0). For example, color development with pentocystine in Series IV is complete in 30 minutes, while with pentocystine in Series 0 only 17 per cent color development was found in 3 hours.

*Series I and II.* —SH Compounds with Sulfite (Figs. 3 and 5)—The reaction of —SH compounds with color reagent in the presence of sulfite (Equations 1 and 3) can be summarized as follows:



Complete double color development (200 per cent) was attained with all the compounds in Series II, yielding the same value of  $\epsilon$ . The rate of color development of most of the —SH compounds in the presence of sulfite resembles that of the corresponding S—S compounds.

With cysteine ester, cysteinylglycine, cysteine, and thioglycolic acid (*cf.* (15)), the reaction of Equation 3 proceeds more slowly than that of Equation 1 (*cf.* particularly the results with thioglycolic acid).

With homocysteine, in both Series I and II, color development is much slower than with homocystine. The presence of lactone in the homocysteine solution may account, at least in part, for this difference. The results with homocysteine indicate that the lactone ring is opened in the presence of sulfite. Pentocystine and hexocystine react more slowly than their corresponding disulfides in the presence of the smaller amount of sulfite (Series I as compared to Series III), while in the presence of the larger amount of sulfite, the rate is the same (Series II and IV).

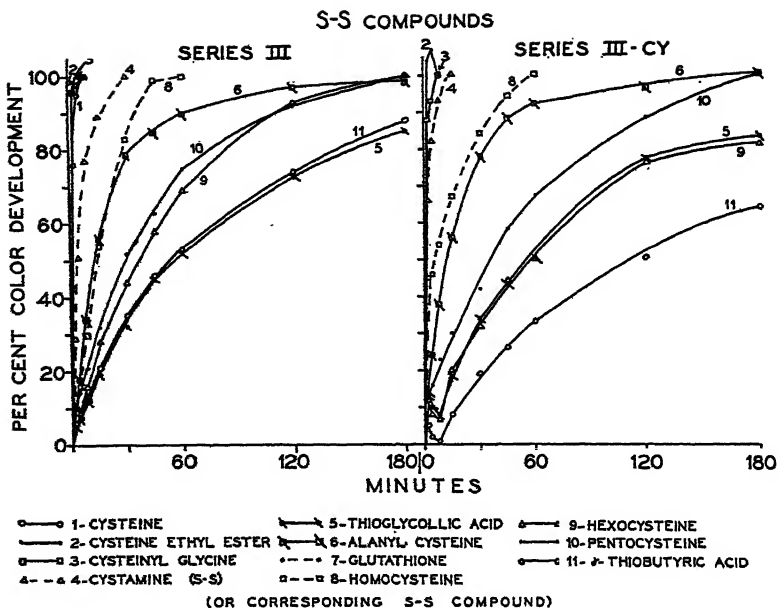


FIG. 7

FIG. 8

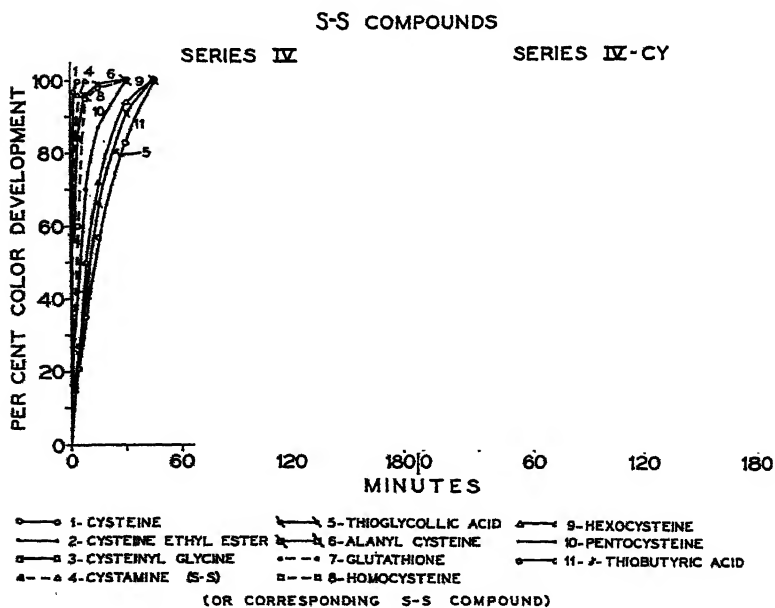


FIG. 9

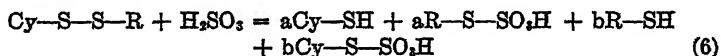
FIG. 10

With glutathione, the rate is considerably increased by the presence of 2 cc. of sulfite, complete double color development being reached in 30 minutes instead of 3 hours.

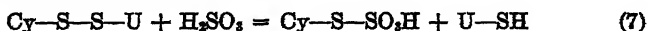
Ergothioneine develops no color in the presence of sulfite.

*Series III-Cy and IV-Cy. S—S Compounds Plus Sulfite in Presence of Cystine (Figs. 8 and 10)*—The reaction of a disulfide ( $R-S-S-R$ ) with sulfite and with color reagent in the presence of cystine ( $Cy-S-S-Cy$ ) is a very complicated process. The first step is obviously the reaction of Equation 3, but the rate of reaction of  $R-S-S-R$  and of  $Cy-S-S-Cy$  with sulfite may be quite different. As  $R-SH$  and  $Cy-SH$  are formed, they will react with the color reagent at different rates (Equation 1), and in addition the reactions between  $-SH$  and  $S-S$  compounds may take place, including the formation of a mixed disulfide ( $Cy-S-S-R$ ).

$Cy-S-S-R$  may react with sulfite at a rate different from  $R-S-S-R$  or  $Cy-S-S-Cy$ , and its decomposition may take place as given in Equation 6.



The relative amounts of the products formed depend on the properties of the group  $R$ , *e.g.* for the mixed disulfide of cysteine and thiourea ( $U-SH$ ), Toennies (13) has shown that the reaction proceeds according to Equation 7,



*i.e.* only cysteinesulfonic acid and thiourea are formed.

In Series IV-Cy color development is faster than in Series IV with all the compounds tested; with dithiodiglycolic acid and hexocystine this is noticeable only in the first few minutes. In Series III-Cy the effect of added cystine on the rate of color development is not uniform. With cystine ester, the color development of (the added) cystine is accelerated. Color development by cystamine and homocystine is accelerated by cystine, that of dialanycystine, pentocystine, and dithiodiglycolic acid is hardly affected, and that of cystinyldiglycine, hexocystine, and dithiodibutyric acid is slowed down. With dithiodiglycolic acid, hexocystine, and dithiodibutyric acid, the apparent decrease in color

development during the first 8 minutes might possibly result from the formation of a mixed disulfide which reacts slowly with sulfite, even more slowly than  $R-S-S-R$  in the last two cases.

*Series I-Cye and II-Cye. —SH Compounds Plus Sulfite in Presence of Cysteine (Figs. 4 and 6)*—The effect of cysteine in accelerating the color development of  $-SH$  compounds is more marked than the effect of cystine on the corresponding  $S-S$  compounds. In Series II-Cye color development with all the compounds is considerably faster than in Series II. In Series I-Cye, color development with cysteine ester is somewhat slowed down, that with thioglycolic acid remains about the same, while all the other  $-SH$  compounds react faster than in the absence of cysteine.

Ergothioneine remains negative in these series and has no effect on the color development by cysteine, indicating that under our conditions no mixed disulfide is formed (the possibility of such a mixed disulfide has been discussed (*cf.* 13)).

*Simultaneous Determination of Two Disulfides*—The results indicate that it is possible to determine two disulfides in the presence of each other, provided that their reaction rates are sufficiently different. In addition to Solution A (*cf.* (12)), Solution  $A_2$  (2 cc. of sulfite<sup>1</sup>) is also prepared and the color in Solution  $A_2$  is allowed to develop for 30 minutes (or longer) before dilution to 50 cc. If only cystine is present  $K(A) = K(A_2)$ , but with a mixture of cystine and a slowly reacting disulfide  $K(A_2)$  will be larger than  $K(A)$ . This procedure has been utilized for the determination of cystine and homocystine in cystinuric urine (*cf.* (6)). The total cystine plus homocystine was found from  $K(A_2)$ , cystine was determined separately by the Lugg-Sullivan method, and homocystine calculated by difference.

#### DISCUSSION

Under suitable conditions of color development, all the compounds tested give the same value (8200) for the molecular extinction coefficient of reduced phospho-18-tungstic acid (Figs. 5 and 9). Differences in color development among the compounds are differences only in rates.

Attempts to calculate velocity constants on the basis of a pseudomonomolecular reaction failed to give uniform results, indicating a complex course of reaction.

In contrast to cystine some other substances of physiological importance, such as ergothioneine, homocystine, and glutathione, develop either no color or considerably less than cystine under the conditions (8 minutes, 0.5 cc. of sulfite) of the photometric cystine method (12). On the other hand cystine derivatives in which only the carboxyl group is substituted give complete color development under these conditions.

High values for the cystine content of proteins have been reported (16-18) with the Folin-Marenzi method after short periods of hydrolysis.<sup>5</sup> On the basis of the results in this paper, it is unlikely that such high values will be found with the photometric method, even if some cystine peptides are still present, owing to incomplete hydrolysis. Either correct or slightly low results will be obtained in such a case, since cystine peptides can be expected to give nearly complete color development in the presence of a large excess of cystine.

#### SUMMARY

1. The rates of reaction of a number of  $\text{—SH}$  and  $\text{S—S}$  compounds with phospho-18-tungstic acid and sulfite have been studied under the conditions of the photometric method. The majority of the compounds tested, including those of physiological importance, react less rapidly than cystine or cysteine. Ergothioneine does not react at all.

2. The addition of cysteine and of cystine to the slowly reacting  $\text{—SH}$  and  $\text{S—S}$  compounds respectively increases the rate of color development in most cases.

3. The reactions which may occur in the presence of two different sulfur compounds have been discussed, and the possible formation of mixed disulfides considered.

4. All the compounds tested, under optimum conditions, give the same value (8200) for the molecular extinction coefficient of reduced phospho-18-tungstic acid.

<sup>5</sup> Color development with cystine is incomplete under the conditions of the Folin-Marenzi method. Since certain peptides, *e.g.* cystinyldiglycine, react faster than cystine, they will develop more color than corresponds to their cystine content. This may explain the high results after short periods of hydrolysis.



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# THE DETERMINATION OF METHIONINE, CYSTEINE, AND SULFATE IN PROTEINS AFTER HYDROLYSIS WITH HYDRIODIC ACID\*

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The hydrolysis of proteins with hydriodic acid has recently been developed by Baernstein (1) into a comprehensive system of analysis of the sulfur amino acids. After considerable experience with these methods, we are submitting recommendations for certain modifications and corrections.

In the course of the hydrolysis with HI we find that the greater part (94 per cent) of the methionine is demethylated with the formation of the lactone of homocysteine (2) and the liberation of methyl iodide, while small amounts of methyl mercaptan and  $\text{H}_2\text{S}$  are also formed. Cystine is almost quantitatively converted into cysteine and only a small part decomposed into  $\text{H}_2\text{S}$  (1 to 2 per cent). The cystine in certain proteins, *e.g.* crystalline insulin (3), may be more labile towards HI than cystine itself. Inorganic sulfate, which is present in many proteins, is reduced to  $\text{H}_2\text{S}$  (or partly to  $\text{SO}_2$ ).

*Apparatus*—The apparatus used differs from that described by Baernstein (1) in the following details. The digestion flask is of 75 cc. capacity with a standard ground joint 19/38, and with a straight 4 inch side tube into which is inserted a hollow sealed glass tube (4). The joint between the digestion flask and condenser is protected by a mercury seal which consists of a rubber stopper with a glass sleeve. The screw clamp at the condenser outlet is omitted and a glass to glass connection to the first ab-

\* This report is from a dissertation submitted by Beatrice Kassel in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

sorber made. It has been found unnecessary to keep the condenser warm. The absorbers are similar to those used by Baernstein, but are open at the top and the side arms reach to the bottom of the next absorber. The side arm of the second absorber contains a glass to glass connection. The absorbers are connected in series by rubber stoppers.

*Reagents*—Reagent quality HI, sp. gr. 1.7, preserved with hypophosphorous acid or potassium hypophosphite is redistilled twice in a stream of  $N_2$  from an all-glass apparatus and the second distillate preserved by the addition of 1 per cent potassium hypophosphite. The HI blank should be determined at weekly intervals. The KI used in the titration should give no blank.

*Digestion*—The Baernstein apparatus was generally used, but for purposes of comparison some experiments were also carried out in the Pregl apparatus.

*Digestion in Pregl Micromethoxyl Apparatus*—For such determinations (0.5 to 5 mg. of methionine) 2 cc. of HI, preserved with only 0.25 per cent of potassium hypophosphite, are used for digestion, 1 cc. of saturated  $HgCl_2$  for the washer, and 3 cc. of 10 per cent potassium acetate in glacial acetic acid plus 3 drops of bromine in the absorber (*cf.* (5)). The digestion is carried out in an oil bath at  $150^\circ$  for 3 to 4 hours, in a stream of  $CO_2$  or  $N_2$ <sup>1</sup> (*cf.* (4)).

The use of HI preserved with 0.25 per cent of hypophosphite and of  $HgCl_2$  in the washer may also be of advantage in micromethoxyl determinations (*cf.* Fig. 1, vanillin).

Pregl's micromethoxyl apparatus in its present form is not convenient for the determination of methionine in proteins on account of the difficulty in introducing larger samples, the possible clogging of the washer during the prolonged heating, and because the analysis is limited to the determination of volatile iodide.

*Digestion in Baernstein Apparatus*—The absorption train is assembled and attached to the condenser. The first absorber contains 10 cc. of a solution containing 20 per cent  $CdCl_2$  with 20 per cent  $BaCl_2$ , the second contains 10 cc. of saturated  $HgCl_2$ , the third 10 cc. of 10 per cent potassium acetate in glacial acetic acid plus 6 drops of bromine, the fourth 5 cc. of the last solution.

The digestion flask with a small porous boiling stone, the hollow tube inserted, and the empty mercury seal attached, is filled with  $CO_2$  or  $N_2$ <sup>1</sup> and the pinch-cock on the side tube closed. 10 cc.

of HI (5 cc. for less than 100 mg. of protein) are now added, wetting the ground joint. The weighing tube containing the substance for analysis is introduced at once, the flask connected to the condenser, and the seal filled with mercury. The flask is placed in an oil bath preheated to  $150^{\circ}$ . After heating 2 to 3 minutes, the pinch-cock on the side tube of the flask is opened and the flow of  $\text{CO}_2$  or  $\text{N}_2$  adjusted so that 3 or 4 bubbles are visible in the absorbers at the same time. Heating at  $150^{\circ} \pm 3^{\circ}$  and aeration are continued for 5 to 6 hours.

The third and fourth (bromine) absorbers are then disconnected at the side arm of the second absorber, the stream of gas being continued. The first two absorbers are disconnected at the condenser outlet, replaced by a second vertical downward condenser, which is kept cold, the speed of the gas stream increased, and the digest concentrated to about 3 cc. over a microburner. The concentrated digest is allowed to cool, the gas stream being continued. At this point the digest is free from hypophosphite and iodine.

*Determination of Volatile Iodide*—In the determination with the Baernstein apparatus, the third and fourth absorbers are washed into a 100 cc. volumetric flask containing 25 cc. of 25 per cent sodium acetate, the bromine removed with formic acid, and the contents diluted to the mark. Aliquots of 30 cc. are treated with 1 to 2 gm. of KI and 5 cc. of 10 per cent HCl, and titrated with 0.01 to 0.05 N thiosulfate.

For the blank determination, the same amount of HI (5 or 10 cc.) as used for the hydrolysis of the protein is digested for 5 to 6 hours. The blank obtained by titrating a 30 cc. aliquot should not exceed 0.1 cc. of 0.01 N thiosulfate. If a higher blank is found, the HI should be redistilled.

In determinations with the Pregl apparatus, the bromine absorber is treated similarly, smaller volumes being used.

1 cc. of 0.01 N thiosulfate is equal to 0.248 mg. of methionine in the aliquot titrated.

The values obtained in the methionine determinations are too low, since methyl mercaptan is formed from methionine (*cf.* below). A correction factor was established with a specially purified preparation of methionine (*cf.* (6)), which gave a satisfactory elementary analysis.<sup>2</sup>

The average recovery of methionine (1 to 20 mg.) in twenty-

eight determinations in the Baernstein apparatus was 93.7 per cent, with a mean deviation of 1.3. The correction factor is therefore 1.067.

In the Pregl apparatus, the recovery of methyl iodide was slightly higher, an average of 94.3 per cent in three determinations with about 3.5 mg. of methionine (*cf.* Fig. 1).

For comparison, a number of volatile iodide determinations were carried out with vanillin, with both the Pregl and the Baernstein apparatus. The average recoveries were 99 and 97 per cent respectively (*cf.* Fig. 1). It therefore seems that in the large apparatus the recovery of volatile iodide is about 2 per cent too low, owing to

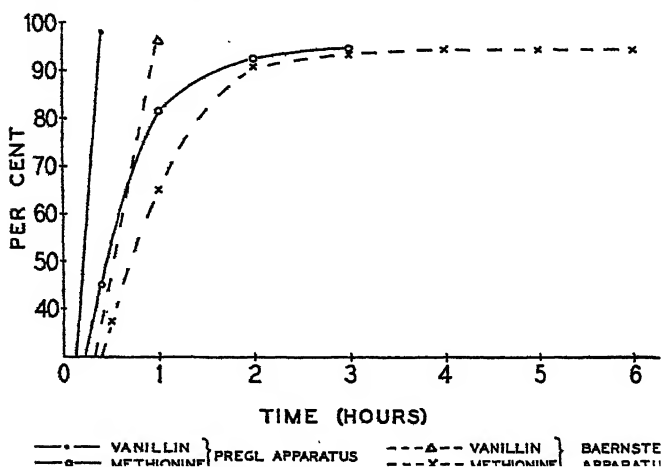


Fig. 1. Liberation of volatile iodide

slight leaks or retention. This 2 per cent and the 3 to 4 per cent of methyl mercaptan formed from methionine together account for the correction of 6 per cent.

*Rate of Liberation of Methyl Iodide*—The rate of liberation of methyl iodide from vanillin and from methionine in the Pregl apparatus and in the Baernstein apparatus is shown in Fig. 1. During the 1st hour of digestion, the liberation of methyl iodide is somewhat slower from proteins than from methionine; the reaction is complete in 5 to 6 hours.

*Determination of Cysteine*—The digest contains cysteine (from cystine and cysteine originally present) and homocysteine lactone

(from methionine). The lactone does not react with iodine.<sup>3</sup> Lavine (7) has shown that the oxidation of cysteine to cystine by iodine proceeds stoichiometrically at room temperature only if the concentration of HI is between 0.5 and 1.5 N; under these conditions an excess of biiodate does not influence the oxidation. However, the excess of biiodate has to be controlled in the cysteine titration, since it may affect the blank in the subsequent determination of homocysteine. It is, therefore, advisable to calculate the amount of cysteine anticipated from the difference between total S and methionine S (from volatile iodide).

The gas stream through the flask containing the concentrated digest is increased and continued at a rapid rate; the mercury is poured from the seal, and the condenser disconnected and rinsed with a few cc. of 4 per cent HCl. The digest is diluted with water to 15 to 20 cc., the temperature adjusted to about 20°, an excess<sup>4</sup> of about 2 cc. of 0.02 N biiodate added, and the excess of iodine titrated with 0.01 or 0.02 N thiosulfate. Since HI digests of protein are slightly yellow, it is necessary to add starch at a point earlier than usual. Near the end-point, as soon as the blue color changes to reddish brown, the solution should be titrated slowly, about 30 seconds being allowed before each drop of thiosulfate is added.<sup>5</sup> In this way a correct end-point is obtained without difficulty.

For the blank determination, the digest is concentrated and treated the same way as the protein digest; 2 cc. of 0.02 N biiodate are added and the mixture is titrated. The blank<sup>6</sup> in the cysteine determination varies from 0 to 0.1 cc. of 0.02 N biiodate.

1 cc. of 0.02 N biiodate is equal to 2.40 mg. of cysteine.

<sup>3</sup> Occasionally, determinations with homocysteine or methionine indicated that a few per cent of the homocysteine were titrated as cysteine.

<sup>4</sup> If the total S or the approximate cystine content of the protein is not known in advance, 0.02 N biiodate is added from a burette until the solution is definitely orange. If much more or much less than 2 cc. of 0.02 N thiosulfate is then required for titration, an adjustment is made in the amount of tetrathionate added in the homocysteine determination.

<sup>5</sup> This procedure is necessary because the starch-iodine complex dissociates slowly in normal HI. We find that it is of no advantage to add excess thiosulfate and titrate to the appearance of the blue color.

<sup>6</sup> Low blanks in the titration of cysteine or other —SH compounds under the conditions established by Lavine (normal HI) can be obtained only if the titrations are carried out in an inert atmosphere.

The correction to be applied in this determination is based upon the results of experiments with pure cystine. In seven experiments in which 5 to 20 mg. of cystine were digested with HI,<sup>7</sup> 97.8 per cent was titrated as cysteine and 1 to 2 per cent as H<sub>2</sub>S. The correction factor<sup>8</sup> for the cystine plus cysteine determination is therefore 1.023.

Occasionally small amounts of homocysteine may be titrated as cysteine; this would be indicated by a discrepancy of more than 2 to 3 per cent between the volatile iodide and the homocysteine determinations. In such cases an appropriate correction may be deducted from the cystine plus cysteine value (cf. Table II, Egg Albumin C). The correction is not appreciable except for proteins which contain large amounts of methionine and comparatively small amounts of cystine. Experiments in which equal amounts of cystine and methionine were digested together gave correct results, but when the ratio of cystine to methionine in the digest was about 1:3 (as in egg albumin) the recovery of cystine was 2 to 5 per cent too high, and that of homocysteine correspondingly low.

*Determination of Homocysteine*—The digest now contains cystine and the lactone of homocysteine. Baernstein (1) has shown that this lactone ring is opened by alkali to yield homocysteine. Tetrathionate quantitatively oxidizes homocysteine to homocystine, while a corresponding amount of thiosulfate is formed, which can be titrated with biiodate.

Since the blank in the homocysteine titration is dependent to a considerable extent on the excess of tetrathionate present, the probable amount of homocysteine in the digest should be calculated in advance from the volatile iodide determination (1 cc. of 0.04 N tetrathionate<sup>9</sup> is equivalent to 6 mg. of methionine).

<sup>7</sup> Cysteine is lost during HCl digestion because of humin formation (cf. (8)). During HI digestion there is no humin formation.

<sup>8</sup> This correction is made on the assumption that the lability of cystine towards HI is the same for cystine in proteins. So far, only one exception has been noted. With three preparations of crystalline insulin (cf. (3)), H<sub>2</sub>S amounting to about 7 per cent of the total S was found. The cystine by the Baernstein method was about 5 per cent less than by the Folin photometric and Sullivan methods.

<sup>9</sup> The solution is prepared by mixing 2 cc. of 0.1 N biiodate with 1 cc. of 10 per cent HCl and a little KI; it is then titrated with 0.1 N thiosulfate, and the end-point adjusted with 0.02 N thiosulfate.

As soon as the cysteine titration is completed, 2 to 3 drops of brom-thymol blue, 1 drop of caprylic alcohol, and the calculated amount of freshly prepared 0.04 N tetrathionate plus an additional 2.5 cc. are added to the digest. (Including the tetrathionate formed during the cysteine titration, the digest should contain an excess of 3.5 cc. of 0.04 N tetrathionate; this makes the tetrathionate concentration about 0.004 N.)

The ground joint of the flask is fitted with a hollow ground glass stopper carrying a connecting tube with a stop-cock which is left open. After the gas is run through for about a minute, the pinch-cock on the side arm and the stop-cock on the connecting tube on top are closed and the gas supply disconnected, enough rubber tubing being left on the side tube to insert a burette. The digestion flask is evacuated through the connecting tube on top until the pressure is about 20 mm., when the stop-cock is closed. Concentrated ammonia (0.5 cc. more than necessary for alkaline reaction) is drawn in through the side tube. The pinch-cock is closed, and the flask evacuated again and allowed to stand at 40° for 15 minutes. 10 cc. of 5 N HCl are drawn in through the side tube, which is then connected to the CO<sub>2</sub> or N<sub>2</sub>, and the flask is filled with gas. The connecting tube on top is opened, taken off, and rinsed with water. With gas bubbling through, the thiosulfate in the digest is immediately titrated with 0.01 or 0.02 N biiodate.

With a total of 3.5 cc. of 0.04 N tetrathionate, the blank usually amounts to 0.3 to 0.5 cc. of 0.01 N biiodate.

1 cc. of 0.01 N biiodate corresponds to 1.49 mg. of methionine.

The recovery of methionine in the homocysteine determination is lower than in the volatile iodide method, as indicated by experiments with pure methionine and with a number of proteins, some of which are reported in Table II. Based upon a series of analyses (Table II) of crystalline Egg Albumin B (a specially purified preparation in which the values for cystine plus cysteine, methionine (as volatile iodide), and sulfate S account for the total sulfur) an empirical correction factor of 1.120 is recommended for the homocysteine determination.

It should be noted that hematin, in contrast to inorganic iron, interferes with the cysteine and homocysteine titrations. These determinations cannot, therefore, be carried out with hemoglobin, but globin gives satisfactory results.



*Determination of H<sub>2</sub>S Sulfur-Sulfate Sulfur*—Sulfate is reduced quantitatively to H<sub>2</sub>S or SO<sub>2</sub>, while small amounts of H<sub>2</sub>S are formed from methionine (0.5 to 1 per cent), cystine (1 to 2 per cent), and homocystine (about 0.5 per cent).

The H<sub>2</sub>S and SO<sub>2</sub> are retained in the first absorber as CdS and BaSO<sub>3</sub>. Experiments with Na<sub>2</sub>SO<sub>4</sub> indicate that no H<sub>2</sub>S passes over into the second absorber. The sulfide and sulfite can be oxidized by iodine to S and SO<sub>4</sub> respectively. An excess (usually 2 cc.) of 0.02 N biiodate is added to the first absorber, followed by about 300 mg. of KI; 2 cc. of 10 per cent HCl are introduced through the inlet tube in order to dissolve any precipitate which may adhere. As soon as the precipitate is dissolved, the excess iodine is titrated with thiosulfate (0.01 to 0.02 N).

The blank is usually 0 to 0.1 cc. of 0.02 N biiodate.

1 cc. of 0.02 N biiodate is equivalent to 0.32 mg. of S.

The correction for sulfate S is made on the basis of our findings with cystine and methionine.<sup>8</sup> From the total H<sub>2</sub>S sulfur, 1 per cent of the methionine S and 2 per cent of the cystine S are deducted.

*Gravimetric Determination of Sulfate Sulfur in Proteins*—300 mg. of protein are hydrolyzed with 10 cc. of 6 N HCl in an oil bath at 130° under N<sub>2</sub> for 6 hours; the hydrolysate is filtered by suction through ashless paper and evaporated to dryness *in vacuo*. The residue is taken up in 1 to 2 cc. of 0.1 N HCl and filtered through a Pregl halogen filter tube with a fritted glass plate into a small test-tube, from which the filtrate and each washing are transferred to a weighed special porcelain crucible (*cf.* (9)). Up to 8 cc. of 0.1 N HCl can be used for washing and transferring. The crucible is heated on a steam bath and 1 cc. of 2 per cent BaCl<sub>2</sub> added drop by drop. Digestion is continued on the steam bath for 1 hour at a somewhat lower temperature, so that not more than 1 to 2 cc. evaporates. The crucible is allowed to stand at room temperature for 2 to 3 hours before filtration. The precipitate is washed three times with about 1 cc. each time of 0.1 N HCl and the crucible plus the filter stick is ignited, cooled, and weighed.

Since BaSO<sub>4</sub> precipitated from protein hydrolysates is frequently contaminated, the purity of the BaSO<sub>4</sub> precipitate is tested by determining its sulfur content. The BaSO<sub>4</sub> precipitate in the crucible is finely powdered with a small glass rod, and a sample of

about 2 mg., but not more than 3 mg., digested in the Baernstein apparatus with 5 cc. of HI for 6 hours as described for methionine (only the  $\text{CdCl}_2$ - $\text{BaCl}_2$  absorber is needed). The  $\text{BaSO}_4$  gradually dissolves and the sulfate is reduced to  $\text{H}_2\text{S}$  (or  $\text{SO}_2$ ), which is titrated in the first absorber, as described under "Determination of  $\text{H}_2\text{S}$  sulfur-sulfate sulfur."

For example, 309.6 mg. of Preparation C of crystalline egg albumin yielded 4.427 mg. of  $\text{BaSO}_4$  equivalent to 0.20 per cent of sulfate S. From the  $\text{BaSO}_4$  precipitate, 2.206 mg. digested with HI gave 0.31 mg. of  $\text{H}_2\text{S}$  sulfur, *i.e.* 14.0 per cent S in the  $\text{BaSO}_4$  (theory 13.7 per cent). The  $\text{BaSO}_4$  in this experiment was therefore pure. However, in other experiments with egg albumin (*cf.* Table III, Preparation B) and other proteins, as much as 15 per cent impurity in the  $\text{BaSO}_4$  has been found, and the value of the sulfate S corrected accordingly.

*Methyl Mercaptan Formation*—The formation of methyl mercaptan from methyl thiol groups has already been observed (10, 11).

If small amounts (0.5 to 2 mg.) of mercury mercaptide are digested with HI in the Baernstein apparatus, within 1 hour the  $\text{CH}_3\text{SH}$  is carried through the first absorber ( $\text{CdCl}_2$ ) and is precipitated by  $\text{HgCl}_2$  in the second absorber<sup>10</sup> (differentiation from  $\text{H}_2\text{S}$ ). The precipitate in the second absorber is a mixture of the mercury salt of  $\text{CH}_3\text{SH}$ , of a mercury compound of phosphine, and of large amounts of  $\text{HgCl}_2$  resulting from the reduction of  $\text{HgCl}_2$  by hypophosphorous acid. Methyl mercaptan can be approximately estimated in this precipitate by dissolving it in sodium cyanide, and distilling under  $\text{N}_2$  into a receiver containing cyanide and zinc acetate (12). The methyl mercaptan in the distillate is determined by means of the nitroprusside reaction, with the Pulfrich photometer and Filter<sup>11</sup> S-53 (Zeiss).

Determinations carried out with varying amounts of methionine (0.7 to 25 mg.) gave results which indicated that 3 to 4 per cent of the methionine was decomposed into  $\text{CH}_3\text{SH}$ . In the HI digestion of proteins,  $\text{CH}_3\text{SH}$  is formed in amounts corresponding

<sup>10</sup> If an extra absorber containing 50 per cent  $\text{ZnCl}_2$  is introduced between the  $\text{CdCl}_2$  and  $\text{HgCl}_2$  absorbers, 70 to 90 per cent of methyl mercaptan is retained by the  $\text{ZnCl}_2$  and can be determined iodimetrically.

<sup>11</sup> This filter has also been used by Schöberl and Ludwig (13).

to about 3 to 4 per cent of their methionine content, as indicated by experiments with casein, egg albumin, edestin, hemocyanin (*cf.* (14)), etc. In these determinations, the odor of mercaptan was clearly perceptible in the cyanide solution.

If methyl mercaptan were produced during HI digestion only from preformed  $\text{S}-\text{CH}_3$  groups, this would be of analytical importance in differentiating  $\text{S}-\text{CH}_3$  from  $\text{O}-\text{CH}_3$ . However, if during HI digestion  $\text{H}_2\text{S}$  is also liberated,  $\text{CH}_3\text{SH}$  may result<sup>12</sup> from the interaction of  $\text{CH}_3\text{I}$  and  $\text{H}_2\text{S}$  (or  $\text{CdS}$ ). Experiments in

TABLE I

*Interaction of  $\text{CH}_3\text{I}$  and  $\text{H}_2\text{S}$  (or  $\text{CdS}$ ) Produced from Vanillin and  $\text{Na}_2\text{SO}_4$  by HI Digestion*

Experiment No.	Vanillin* mg.	$\text{Na}_2\text{SO}_4$ as S mg.	Time of digestion hrs.	Mercaptan test	$\text{CH}_3\text{I}$ and $\text{H}_2\text{S}$ in amounts equivalent to
1	1.48	0.406	2	Negative	200 mg. insulin
2	13.46	0.151	2	"	500 " lactalbumin ( <i>cf.</i> Tables II and III)
3	25.68	1.004	2	++	500 mg. Egg Albumin C ( <i>cf.</i> Tables II and III)
4	25.43	1.174	5	+++†	500 mg. Egg Albumin B ( <i>cf.</i> Tables II and III)
5	25.26	1.171	6	Negative	500 mg. Egg Albumin B
6	11.70	0.727	1	+	

\* 1 mg. of vanillin is equivalent to 0.98 mg. of methionine.

† The extinction coefficient was equivalent to 0.15 mg. of mercury methyl mercaptide  $\approx$  0.15 mg. of methionine.

which vanillin and  $\text{Na}_2\text{SO}_4$  were digested together are reported in Table I. It can be seen that in certain experiments (Nos. 3, 4, and 6) traces of methyl mercaptan were formed.

It, therefore, seems probable that most of the methyl mercaptan found after HI digestion of proteins results from the decomposition of methionine, but with proteins which contain sulfate, small amounts of methyl mercaptan may be formed by a secondary reaction between  $\text{CH}_3\text{I}$  and  $\text{H}_2\text{S}$  (or  $\text{CdS}$ ).

<sup>12</sup> This was suggested as a possibility by Dr. V. du Vigneaud.

TABLE II

*Determination of Methionine and of Cystine Plus Cysteine in Proteins*

Protein preparation	Sample	Methionine				Cystine + cysteine	
		Volatile iodide		Homocysteine		Found	Corrected, $F = 1.023$
		Found	Corrected, $F^* = 1.067$	Found	Corrected, $F = 1.120$		
	mg.	per cent	per cent	per cent	per cent	per cent	per cent
Crystalline Egg Albumin A	306.6	4.92	5.25	4.72	5.28	1.68	1.72
Crystalline Egg Albumin B	522.0	4.91	5.24	4.71	5.28	1.73	1.77
	509.5			4.58	5.14	1.72	1.76
	429.6	4.91	5.24	4.77	5.34	1.73	1.77
	427.1	4.88	5.21	4.63	5.20	1.78	1.82
Average.....		4.90	5.23	4.67	5.23	1.74	1.78
Crystalline Egg Albumin C	390.8	4.92	5.25	4.56	5.11	1.82	1.86
	424.5	4.87	5.19	4.60	5.15	1.81	1.85
	382.3	4.89	5.22	4.54	5.09	1.89	1.93
	311.0	4.88	5.21	4.56	5.11	1.87	1.91
	313.8			4.47	5.01	1.83	1.87
Average.....		4.89	5.22	4.55	5.10	1.85	1.89†
Lactalbumin (Labco 7-HAAX)	480.9	2.67	2.85	2.62	2.93	3.03	3.09
	495.6	2.63	2.81	2.54	2.84	3.02	3.08
	528.0	2.59	2.76				
Average.....		2.63	2.81	2.58	2.89	3.03	3.09
Same	205.6	2.67	2.85				
	103.4	2.48	2.65				
	27.0	2.52	2.69				
Casein (Labco)	515.9	2.99	3.21	2.75	3.08	0.38	0.39
	510.8	2.88	3.09	2.71	3.04		
	501.1	2.99	3.21				
Average.....		2.95	3.17	2.73	3.06	0.38	0.39

\*  $F$  = correction factor.

† 1.79 per cent corrected (volatile iodide (5.22) minus homocysteine (5.10) = 0.12, equivalent to 0.10 cystine).

*Results*

Some representative results with proteins are given in Tables II to IV (other data will be published separately). The casein and

TABLE III  
*Determination of Sulfate S in Proteins by H<sub>2</sub>S Titration and BaSO<sub>4</sub> Precipitation*

Protein preparation	Sample	H <sub>2</sub> S sulfur				Sulfate S, (BaSO <sub>4</sub> )	
		Total found	Correc- tion for methi- onine*	Correc- tion for cyst- ine†	Sulfate S (1) - (2 + 3)	Found	Cor- rected
		(1)	(2)	(3)	(4)		
	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Crystalline Egg Albumin A	289.8					0.00	
	306.6	0.022	0.011	0.009	0.00		
Crystalline Egg Albumin B	310.6					0.27	0.23
	290.2					0.26	0.24
	509.5	0.24	0.011	0.009	0.22		
	429.6	0.21	0.011	0.009	0.19		
	427.1	0.20	0.011	0.009	0.18		
	534.9	0.22	0.011	0.009	0.20		
Average.....					0.20		0.24
Crystalline Egg Albumin C	309.6					0.20	0.20
	390.8	0.15	0.011	0.009	0.13		
	424.5	0.14	0.011	0.009	0.12		
	382.3	0.18	0.011	0.009	0.16		
	311.0	0.15	0.011	0.009	0.13		
	313.8	0.15	0.011	0.009	0.13		
Average.....					0.13		0.20
Lactalbumin (Labco)	141.0					0.00	
	480.9	0.032	0.006	0.016	0.01		
	495.6	0.030	0.006	0.016	0.01		
	528.0	0.035	0.006	0.016	0.01		

\* 1 per cent of methionine S.

† 2 per cent of cystine S.

lactalbumin are Labco products obtained through the courtesy of Dr. G. C. Supplee. Crystalline Egg Albumin A was prepared according to Kekwick and Cannan (15), recrystallized three

times, and dialyzed against distilled water with the addition of ammonia (*cf.* (16)), until it was entirely free from sulfate. The egg albumin solution was evaporated to dryness *in vacuo* over  $\text{CaCl}_2$  at room temperature; the solid protein then contained about 10 per cent of the water. For Preparations B and C of denatured crystalline egg albumin, we are indebted to Dr. H. O. Calvery (*cf.* (17)). These preparations contained small amounts of sulfate; Preparation B had been recrystallized repeatedly, Preparation C only once. Before analysis, the proteins were extracted overnight in a Soxhlet apparatus with petroleum ether and dried at  $100^\circ$  *in vacuo*.

TABLE IV

*Sulfur Distribution in Crystalline Egg Albumin and in Lactalbumin*

The values are given in per cent.

Protein preparation	Cystine + cyst- eine S	Methionine S		Sulfate S			Total S		
		Volatile iodide	Homo- cysteine	As H <sub>2</sub> S	As BaSO <sub>4</sub>	+ (1 + 2)	Found (Fregl <sup>o</sup> )	(1 + 2 + 4)	(1 + 2 + 6)
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Egg Albumin A	0.46	1.13	1.14	0.00	0.00	0.01	1.60	1.59	1.59
“ “ B	0.47	1.12	1.12	0.20	0.24	0.26	1.85	1.79	1.83
Lactalbumin	0.83	0.60	0.62	0.01	0.00	0.00	1.42	1.44	1.43

\* Determined according to the modification of Saschek (9), except for ignition of the  $\text{BaSO}_4$ .

It can be seen from Table II that individual determinations of volatile iodide, homocysteine, and cystine plus cysteine do not differ from the average of several determinations by more than 2 per cent. With a very small amount of lactalbumin (equivalent to 0.8 mg. of methionine), the deviation is about 4 per cent. The determinations of  $\text{H}_2\text{S}$  sulfur (Table III) agree within 0.03 per cent of S.

In general, the results obtained for methionine are more consistent by the volatile iodide<sup>13</sup> than by the homocysteine method.

<sup>13</sup> High results may conceivably be obtained if the protein contains a carbohydrate, which yields volatile iodide on digestion with HI. However, *glucose* does not interfere, since 35 mg. gave only a trace of volatile iodide (equivalent to 0.03 mg. of methionine).

With egg albumin,<sup>14</sup> identical values were always obtained by the volatile iodide method (with the three preparations in Table II and others not reported). The homocysteine titration, however, sometimes gave low results, with correspondingly high results for cystine plus cysteine (Preparation C and others). This is apparently due to the titration of a small amount of homocysteine as cysteine (*cf.* above under "Determination of cysteine"). In such a case the value for cystine plus cysteine should be corrected, as indicated for Preparation C in Table II. For similar reasons, the value of 2.02 per cent, reported by Baernstein (1), for the cystine content of egg albumin is probably too high.

The cystine content of lactalbumin reported in Table II is the same as that found by the Folin photometric and Sullivan methods (*cf.* (19)). The values for sulfate S determined as  $H_2S$  (Table III, Column 4), as  $BaSO_4$  (Column 6), and by difference (Table IV, Column 6) are in fair agreement. In Table IV, the sulfur distribution in two preparations of crystalline egg albumin and in lactalbumin is presented. The total sulfur is accounted for within the limits of error.

With a protein of average S content, *e.g.*, lactalbumin, a total of about 150 mg. is sufficient for HCl hydrolysis (microphotometric determination of cystine and of cysteine (19)), for HI hydrolysis (volatile iodide, homocysteine, cystine plus cysteine, sulfate S), and for the determination of total S (Pregl).

#### SUMMARY

1. The Baernstein method for the determination of the sulfur amino acids has been studied in detail and certain modifications in the procedure are suggested.

2. Corrections have been established for the determination of methionine (volatile iodide, homocysteine), of cystine plus cysteine, and of sulfate S (as  $H_2S$ ).

3. Small amounts of methyl mercaptan are formed during HI

<sup>14</sup> Five cysteine residues per molecule of egg albumin (molecular weight = 34,300) correspond to 1.76 per cent and 12 methionine residues to 5.22 per cent. Since a ratio of 5 does not fit into the theory recently developed by Bergmann and Niemann (18), it is possible that in egg albumin the frequency of cystine and cysteine residues must be calculated separately.

digestion of methionine. It may also be formed in traces by the interaction of  $\text{CH}_3\text{I}$  and  $\text{H}_2\text{S}$  (or  $\text{CdS}$ ).

4. A microgravimetric procedure is described for the determination of sulfate S in proteins. The purity of the  $\text{BaSO}_4$  is checked by converting it to  $\text{H}_2\text{S}$ , which is determined iodometrically.

5. Representative results are given with crystalline egg albumin, lactalbumin, and casein. Within the limits of error, methionine, cystine plus cysteine, and sulfate account for the total S in these proteins.

6. The distribution of the sulfur in a protein (of average S content) can be established with a total of about 150 mg.; the total S is determined by the Pregl method and a hydrolysis is carried out with both  $\text{HCl}$  and  $\text{HI}$ , with the use of the Folin microphotometric method for cystine and cysteine, and the procedures described in this paper for methionine, cystine plus cysteine, and sulfate.

7. A procedure is suggested for the determination of methoxyl groups with the Pregl apparatus, with purified  $\text{HI}$  preserved with hypophosphite for digestion and a saturated  $\text{HgCl}_2$  solution in the washer.

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## STUDIES IN GASTRIC SECRETION

### VI. A STATISTICAL ANALYSIS OF THE NEUTRAL CHLORIDE-HYDROCHLORIC ACID RELATION IN GASTRIC JUICE\*

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(Received for publication, June 16, 1938)

In a previous contribution to this subject (Hollander, 1932), it was reported that when the neutral chloride concentration of pure gastric juice from dogs with fundic pouches was plotted against the total acidity, the resulting graph was a straight line. On extrapolation, the intercept of this line with the acidity axis (designated as the acidity-intercept) was found to possess a mean value of 167 mm; for the six sets of data cited, the individual values ranged from 163 to 173 mm. Physiologically, this means that a specimen of gastric juice of this acidity contains no neutral chloride. Also, it was shown that the acidity of such a fluid is *practically constant and isosmotic with mammalian blood*. Consequently, from this and evidence previously obtained, it was inferred that this theoretical base-free isotonic specimen of gastric juice probably corresponds to the pure acid secretion from the parietal cells. According to this view, the fixed base which always occurs in gastric juice is derived from one or more non-acid secretions, wherein it exists either as neutral chloride or as bicarbonate and phosphate which become converted to neutral chloride on admixture with the HCl from the parietal cells. It is important to note that the straight line relations of the foregoing investigation were all deduced from the data by a graphic method which, there was every reason to believe, was reliable for the purpose.

Subsequently, a similar investigation of acidity-chloride relations in gastric juice was reported by Liu, Yuan, and Lim (1934).

\* This work was supported in part by a grant from the Friedsam Foundation.

This study was based on experiments on dogs with fundic pouches and on animals subjected to sham feeding (gastric fistula); it differed from my own in these two major respects: it included the volume of each specimen of secretion, and the data were analyzed by a reliable statistical procedure rather than by a graphic method. As a result of this analysis, these authors found the following: (1) The mathematical relation between the observed output (*i.e.*, the product of concentration and volume) of total acidity in any specimen and the volume of that specimen is best represented by a straight line with positive slope and a value for the intercept on the volume axis which is positive and of significant magnitude. The same is true for the relation between total chloride output and volume. (2) By derivation from the foregoing, the relation between the concentration of total chloride and the volume is represented by a hyperbolic equation, the constants for which were also evaluated statistically. (3) Finally, from these hyperbolic equations, these authors were able to deduce a linear relationship between neutral chloride and acid concentrations similar to that which had been reported by me. However, by inference from their equations they concluded that the acidity-intercept "must necessarily be greater than 176 mm," whereas the average value for the intercept which I found by graphic means was only 167 mm. Hence, these investigators were in agreement with my interpretation concerning the constancy of the HCl concentration in the parietal secretion, but they found this fluid to be hypertonic with respect to blood, rather than isotonic as I suggested. In an effort to account for this discrepancy they point out that a graphic method for determining the acidity-intercept is less reliable than one based on the least squares principle, that a similar statistical analysis of my data might reveal far better agreement with their own results than is now apparent, and therefore that the close numerical agreement between my hypothesis and my observations may be entirely accidental.

Now, the observations and interpretations of these two studies are basic to all further work done in an effort to understand the chemical processes involved in gastric secretion. It was therefore incumbent upon me to reexamine my own data by an accepted statistical procedure and thus to determine the most reliable values for the acidity-intercepts. The present report is a summary of

such an analysis of the several series of data which were previously studied graphically. It also includes several possible causes to account for the difference between the acidity-intercept values arrived at in the two investigations.

### Statistics

In the original investigation, graphic analyses were made of six independent sets of data, each of which correlated total chloride concentration with total acidity and neutral chloride concentra-

TABLE I  
*Summary and Statistical Analysis of Data; Comparison of Experiments*

	B-57	19 experi- ments	C-19	MacLean	C-20	C-25
$N$	13	121	31	16	14	20
$\bar{X}$	45.5	39.8	133.4	110.1	19.2	42.3
$\bar{Y}$	22.5	18.8	31.8	44.0	45.6	12.5
$\mu_{xy}$	853.6	646.7	4011.2	4266.9	726.8	462.0
$m_1$	-0.811	-0.805	-0.746	-0.814	-0.904	-0.377
$b_1$	59.3	50.9	131.3	133.5	63.0	28.4
$m_2$	-1.216	-0.874	-1.280	-1.213	-1.035	-1.726
$b_2$	72.8	57.2	174.1	163.5	66.5	63.8
$r_{xy}$	-0.993	-0.839	-0.977	-0.994	-0.967	-0.807
$\sigma_x$	$\pm 14.4$	$\pm 12.0$	$\pm 18.0$	$\pm 26.5$	$\pm 12.9$	$\pm 13.2$
$\sigma_y$	$\pm 11.3$	$\pm 10.5$	$\pm 13.4$	$\pm 21.7$	$\pm 12.0$	$\pm 6.2$
$S_x$	$\pm 1.2$	$\pm 6.5$	$\pm 3.8$	$\pm 3.0$	$\pm 3.3$	$\pm 7.8$
$S_y$	$\pm 1.0$	$\pm 5.7$	$\pm 2.8$	$\pm 2.4$	$\pm 3.0$	$\pm 3.7$

$\bar{Y}$  = average value of neutral chloride concentration

$\bar{X}$  = average value of (total acidity minus  $k$ ) where  $k$  is a parameter, introduced only to simplify the calculations

$b_2$  = (the true acidity-intercept minus  $k$ )

tion with total acidity. The conditions under which these data were collected need not be discussed here, since they were fully described previously. Our present concern is chiefly with the neutral chloride relations, and therefore this statistical study is based upon the linear relation between this latter variable and the acidity. However, the neutral chloride values were determined as the difference between total chloride and total acidity; but it can be shown that the results for the neutral chloride relations can be carried over to the total chloride relations as well. Since

the argument gains nothing by this additional calculation, the latter has not been included in the present analysis.

The statistical method employed was that given by Richardson (1934). A summary of the data and calculations is presented in Table I.

### *Interpretation*

Our main problems may be stated as follows: (1) How reliable was the visual (graphic) estimate of a straight line relation between the two variables? (2) What is the most probable value of the acidity for which, on extrapolation, the neutral chloride value becomes zero; *i.e.*, of the acidity-intercept?

TABLE II  
*Acidity-Intercept Values ( $I_A$ ) Obtained Graphically and by Calculation*

Experiment	$N$	$I_A$ (graphic)	$I_A$ (calculated)*	$\pm S_x$	$r_{xy}$
		<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
B-57	13	168	167.8	1.2	-0.99
19 experiments	121	167	157.2	6.5	-0.84
C-19	31	173	174.1	3.8	-0.98
MacLean	16	163	163.5	3.0	-0.99
C-20	14	173	164.5	3.3	-0.97
C-25	20	166	163.8	7.8	-0.81

\* Calculated from the line of regression of  $X$  on  $Y$ .

Concerning the reliability of the linear relation between total acidity and neutral chloride, as estimated visually, no quantitative statement can be made. For the calculated regression lines, however, the goodness of fit is given by the correlation coefficient,  $r_{xy}$ . Now, for two of the six experiments under consideration, the values of  $r_{xy}$  were found to be -0.84 and -0.81 (Table II), both of which are sufficiently large to indicate fairly good linear correlation. For the other four experiments, the correlation is even better, as indicated by  $r_{xy}$  values of -0.99, -0.98, -0.99, and -0.97 respectively. These values are so very high that they leave no doubt of the validity of the linear relations presumed in the above analysis.

To answer the second question we must determine the acidity-

intercept of the regression line obtained by making the neutral chloride concentration the independent variable. The equation for this line is

$$X = m_2Y + b_2 \quad (1)$$

Defining  $I_A$  as the acidity-intercept, which is given by the value of  $X$  when  $Y = 0$ , we have

$$I_A = b_2 \quad (2)$$

The values of  $I_A$  together with their standard errors,  $S_x$ , are given in Table II, where they can be compared with the corresponding values of  $I_A$  arrived at graphically. In four cases, the difference between the graphic value and that obtained by calculation is so small as to be negligible in comparison with  $S_x$ ; i.e., 0.2, 1.1, 0.5, and 2.2 mm. In the two other experiments, the differences are considerable, 9.8 and 8.5 mm, but in both instances the  $I_A$  values arrived at statistically are less than the graphic ones—not greater than them, as Liu and his coworkers were led to expect by their data. Furthermore, the arithmetical mean of these  $I_A$  values is 165.2 mm, with a standard deviation of  $\pm 2.3$  mm (as compared with 167 mm determined by graphic measurement), whereas the mean value reported by Liu *et al.* is around 176 mm. Thus the mean intercept values arrived at statistically and graphically are in essential agreement with each other; in fact, the former value differs from that of Liu *et al.* even more than does the latter.

Finally, let us compare the mean value which has just been arrived at for the concentration of HCl in the parietal secretion with the value reported by Liu *et al.*, in order to determine whether or not the difference between them is significant statistically. For this purpose, let us apply the usual test for significance of a difference of two means ( $M_1$  and  $M_2$ ) in terms of their standard deviations. This test is based on the formula,

$$m = \frac{(M_1 - M_2)}{\sigma_{(M_1 - M_2)}} = \frac{(M_1 - M_2)}{\sqrt{\sigma_{M_1}^2 + \sigma_{M_2}^2}} \quad (3)$$

If  $m$  is greater than 3, the difference is certainly significant (with a probability of over 99.7 in 100); if  $m$  is less than 2, the probability of its being significant is low (less than 95 in 100). For values

between 2 and 3, therefore,  $(M_1 - M_2)$  may or may not be significant.

In the present situation,  $M_1 = 176$ ,  $M_2 = 165.2$ ,  $\sigma_{M_1} = 1.9$  (calculated from the data of Liu *et al.* by the usual method), and  $\sigma_{M_2} = 2.3$ . Substituting in Equation 3 we find that  $m = 3.6$ . From this we must conclude that the difference between my determination of the acidity-intercept value and that of Liu *et al.* is probably significant. Why this difference occurs is not immediately apparent, but several possible contributing causes suggest themselves.

1. Its existence may be a matter of difference in physiological state, related to water balance, of the individual animals employed in the two investigations.

2. Also, the difference in acidity-intercept values may result from specific differences in experimental procedure. For instance, Liu *et al.* measured three variables: total acidity, total chloride, and volume of secretion. All three of these variables entered into the evaluation of their constants for the straight line relation, although the volume variable itself does not appear in this relation. Now, there is a systematic error in determining the volume of secretion—an error which arises from the retention of small amounts of the fluid within the stomach (or pouch) and the device used for collecting the gastric juice. In the case of small volumes, i.e. those less than 10 cc., the percentage magnitude of this error may be considerable, and the error is always in the same direction. In order to eliminate this particular source of error, their acidity intercepts should be redetermined directly from the acidity and chloride values, with exclusion of the volume of secretion.

3. Another difference in experimental procedure employed in our respective studies may also be significant in this connection. To determine total acidity, Liu *et al.* used phenolphthalein as a titration indicator—presumably to a rose color, which corresponds to a pH around 9.0. On the other hand, my end-point was pH 7.0, determined by comparison with a buffer standard at this pH value. Since gastric juice possesses a measurable though small buffer capacity, it follows that their titration values must be uniformly greater than mine. In order to verify this, a dozen specimens of gastric juice from dogs and humans were titrated to definite end-points at pH 3.5, 7.0, and 9.0 by our customary technique (Hollander, 1931). The indicators were brom-phenol blue (free

acidity), phenol red (total acidity), and phenolphthalein (total acidity according to Liu *et al.*). The results for these miscellaneous specimens of widely varying acidity are shown in Table III. The total acidity values as estimated by the two methods differ by 2 to 11 mM (average, 5 mM) for a mean total acidity of 80 mM. Thus, this difference in experimental procedure may be held responsible for perhaps half of the difference between our respective mean acidity-intercept values (10.8 mM). Based on the theory of titration of buffer-containing solutions, there can be no doubt

TABLE III

*Titer of Gastric Juice to Various End-Points (Expressed As mM Concentration of HCl)*

Specimen No.	pH of end-point			Difference between pH 7.0 and 9.0
	3.5	7.0	9.0	
	mM	mM	mM	
1	158	160	163	3
2		23	34	11
3	8	22	30	8
4		9	16	7
5	141	144	146	2
6	150	154	158	4
7	71	76	82	6
8	152	155	157	2
9	76	80	82	2
10	79	25	31	6
11	26	40	47	7
12	58	67	71	4
Mean.....	77	80	85	5

that an end-point of 7.0, precisely determined by comparison with a buffer standard, is more correct than an end-point of considerably higher pH value and determined by a gross color change. To this extent, therefore, the acidity-intercept value determined with phenol red is more reliable than the one determined with phenolphthalein.

4. Finally, in fitting a straight line to their output-volume ( $Q$ - $V$ ) data, Liu *et al.* determined only one regression line, that of  $Q$  on  $V$ . This line yields the most probable value of the output (of total acidity or of total chloride) for an assigned value of the



volume. No reason for the choice of this regression line over the other (that of  $V$  on  $Q$ ) is given, nor is any such reason apparent from the context—particularly since the present situation specifically demands the most probable value of *acid concentration* for an assigned value of *neutral chloride concentration*. It is certainly probable that, were their constants  $a$  and  $b$  evaluated from this other line of regression, the resulting mean value for  $b_{Cl}$  would be appreciably different from 176 mm, and possibly in better agreement with the value computed in this study.

#### SUMMARY

1. The linear relation between total acidity and neutral chloride concentration in gastric secretion of the dog which was previously demonstrated by a graphic procedure has been confirmed here by a statistical analysis of the data. The correlation coefficients for two of the six sets of data are  $-0.81$  and  $-0.84$ . For the other four sets of data the coefficients are all in the range  $-0.97$  to  $-0.99$ .

2. The weighted mean of the acidity-intercept values ( $165.2 \pm 2.3$  mm), calculated from the equations for these regression lines, is statistically identical with the mean value arrived at graphically (167 mm).

3. Although this value differs from that calculated by Liu, Yuan, and Lim from their own observations by about 6 per cent, the difference between them has been shown to be significant statistically. Several possible causes which contribute to this difference are suggested.

4. Thus, the statistical evidence confirms the view previously formulated in this series of studies, that pure parietal secretion is approximately isotonic with the tissue fluids, rather than *hyper-tonic* as claimed by Liu, Yuan, and Lim. This evidence refutes the suggestion advanced by these investigators that the agreement between observation and hypothesis which was previously reported is "accidental rather than real."

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# EXPERIMENTS WITH A FACTOR PROMOTING GROWTH AND PREVENTING PARALYSIS IN CHICKS ON A SIMPLIFIED DIET\*

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When chicks receive a simplified diet containing concentrates of the known vitamins, growth is slow, and the birds often develop a fatal paralysis. Some experimental findings relative to this observation were reviewed previously (1). The present communication reports further studies with chicks fed a simplified diet (2).

## EXPERIMENTAL

The basal diet (Diet 101) had the following composition.

Corn-starch.....	65
Sardine meal, fat-extracted.....	25
Aqueous extract of rice bran (3, 4).....	7
Whey adsorbate.....	2
Cod liver oil.....	1
Manganous sulfate, commercial anhydrous.....	0.1
Hexane extract of alfalfa meal, equivalent to 1 per cent of alfalfa meal, evaporated on the diet	

The diet is the same as previously described (2) except for an addition of manganous sulfate to prevent slipped tendons (5). 0.03 per cent of manganous sulfate was not sufficient for this purpose, but 0.1 per cent gave complete protection. The rice bran extract contained 50 international units of thiamine per cc. and was found by assay with chicks to contain 20 units of filtrate factor per gm. (6). Rice bran extract is also a good source of

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the P-P factor (7) and the rat antidermatitis factor (4), although it is not yet known whether these factors are needed by chicks. The whey adsorbate (6, 8) supplied about 70 chick units of riboflavin per gm. (9). 1 chick unit corresponds to about 2.7 micrograms of riboflavin (unpublished data). The hexane extract of alfalfa meal was of known vitamin K potency, and was furnished by Dr. H. J. Almquist. Sardine meal was used because it was found to give growth superior to that induced by casein when added to a diet containing all known nutritional essentials other than protein. A similar result was noted by Almquist, Stokstad, and Halbrook (10). A preliminary depletion period was not used, because better experimental results were obtained if the chicks were placed on the test diets as soon as they were hatched. A similar observation was made by Pappenheimer and Goettsch (11). Details concerning the chicks and their care have been described previously (12).

Chicks on basal Diet 101 grew fairly well for 2 weeks, after which their growth rate was subnormal for the next week or two. Symptoms of paralysis began to appear in some individuals during the 4th week. A typically affected chick would lose weight for 2 or 3 days, and paralytic symptoms would then appear. The chick would run for a few steps, and then sink on its hocks. In the next stage, the chick would be unable to stand, but could right itself when pushed on its side. In the final stage the chick would lie on its side with its legs extended. Death usually took place within a day. Many individuals, however, died, often during the night, without paralytic symptoms being observed. Still others died on the day on which indications of the first stage of paralytic symptoms were observed. A loss in weight always preceded death. The greatest incidence of symptoms and mortality was typically during the 4th and 5th weeks, although some individuals survived for 7 weeks or even longer. The individual variation in susceptibility was very marked.

The experimental period was usually 5 to 6 weeks, although in some experiments the early appearance of paralytic symptoms in chicks on the basal diet made the results apparent sooner than this. Protection against the symptoms was first observed with a supplement of soy bean oil or with the unsaponifiable matter prepared from it (2). Growth, however, was subnormal on the

supplemented diets. In the first experiment, an attempt was made to find the optimal level of soy bean oil, with the results shown in Table I.

The "normal" weight at 37 days for this strain of chicks was found to be in the neighborhood of 360 gm. Hence none of the experimental groups grew satisfactorily, and levels of soy bean oil higher than 10 per cent apparently exerted a deleterious effect upon growth. In the next experiment a search for a more effective source of the protective factor was initiated. The results are illustrated in Fig. 1.

The addition of peanut meal induced better growth but gave poorer protection against paralysis than the addition of soy bean

TABLE I

*Results Obtained at 37 Days on Chicks Fed Diet 101 with Supplements of Varying Levels of Soy Bean Oil*

Group No.	Supplement	No. of chicks	No. of survivors	Average weight of survivors	No. of observed cases of paralysis
				gm.	
1	None	20	8	115	4
2	5% Soy Bean Oil 1	16	10	154	3
3	10% " " " 1	16	12	174	1
4	15% " " " 1	16	11	136	0
5	20% " " " 1	16	14	104	0
6	10% " " " 2	16	12	136	0

oil. This may indicate that soy bean oil has a depressing effect on growth, or it may indicate that the peanut meal was relatively rich in a growth-promoting factor, but poor in a factor which protected against paralysis. The results indicated that alfalfa meal was an excellent source of the antiparalytic factor and also stimulated growth. The next experiment was devoted to an attempt to investigate the possible fat solubility of the active factor in alfalfa meal. It has been shown by Almquist and Stokstad (13) that hexane will extract vitamin K and the gizzard factor from alfalfa meal. It was thought desirable to test alfalfa hexane extract and residue for antiparalytic potency. Included in the experiment was a group of chicks on a normal diet (14) treated with a solution of ferric chloride in ether, since the results of

Adamstone (14) suggested that the external symptoms observed by him might be similar to those in the present investigation. The results given in Table II were obtained.

At 34 days, the three survivors in Group 11 averaged 80 gm. in weight. The massive dose of alfalfa hexane extract fed to Group 12 produced a definite, but slight, alleviation of the symptoms. On the other hand, the residue from hexane extraction protected

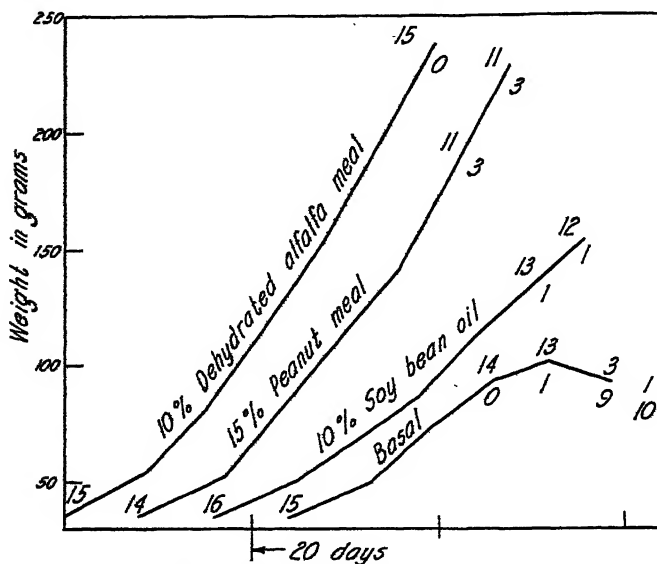


FIG. 1. Growth of chicks on basal Diet 101 compared with growth obtained on Diet 101 *plus* various supplements. The numbers above the growth curve represent the number of chicks surviving in each group; at the beginning of the curves, the number of chicks originally placed in the group; below the curves, the number of observed cases of paralytic symptoms.

completely. Growth was very slow on the ferric chloride-treated diet. At 60 days, the chicks in this group averaged 360 gm. in weight. The chicks in this group were given a weekly dose of 0.1 cc. of shark liver oil containing 40,000 international units of vitamin A per gm.<sup>1</sup> One case of paralysis appeared on the 57th day.

<sup>1</sup> Kindly furnished by Mr. T. D. Sanford, F. E. Booth Company, San Francisco.

In the next experiment, 1 kilo of hexane-extracted alfalfa meal was treated with two successive 8 liter portions of water at 60°. The aqueous extract was strained through cloth and clarified by centrifuging. It was concentrated at 40° under reduced pressure

TABLE II

*Results Obtained at 42 Days on Chicks Fed Diet 101 with Supplements of Alfalfa Meal and Extracts Therefrom*

Group No.	Ration	No. of chicks	No. of survivors	Average weight of survivors	No. of observed cases of paralysis
				gm.	
11	Diet 101 (basal)	12	0		5
12	" 101 + hexane extract of alfalfa meal, equivalent to 40% alfalfa meal, evaporated on diet	12	5	136	5
13	Diet 101 + 10% hexane-extracted alfalfa meal	12	12	266	0
14	Diet 101 + 10% alfalfa meal	12	12	289	0
15	Normal diet (1) treated with ferric chloride	12	12	147	0

TABLE III

*Results Obtained at 33 Days on Chicks Fed Various Supplements to Diet 101*

Group No.	Supplement	No. of chicks	No. of survivors	Average weight of survivors	No. of observed cases of paralysis
				gm.	
16	None (basal diet)	14	5	99	4
17	10% hexane-extracted alfalfa meal	12	11	202	0
18	Water extract of 10% hexane-extracted alfalfa meal	12	11	192	0
19	Water-insoluble residue of preceding	12	6	166	4
20	0.015% nicotinic acid amide (lowered to 0.0075% at 15 days)	10	0		1

until 1 cc. was equivalent to 3 gm. of hexane-extracted alfalfa meal (Water Extract 1). The residue from water extraction was not washed. It was dried at 40°. A group of chicks was included which received 0.015 per cent of nicotinic acid amide as a supple-

ment because of the lack of knowledge of the rôle of this compound in the nutrition of the chick. The results given in Table III were obtained.

The results indicated that the protective factor was easily extracted by warm water. The group receiving nicotinic acid amide gained an average of only 12 gm. in the first 15 days compared with 28 gm. for the group on the basal diet. The level of nicotinic acid amide was therefore halved, but all the chicks were dead by the 29th day.

A potent water extract of hexane-extracted alfalfa meal was submitted to fractionation in the next experiment by some of the methods applied to yeast by Kinnersley and coworkers (15). 1 kilo of hexane-extracted alfalfa was twice extracted for 2 hours at 60-70° with 8 liters of water. Each time the suspension was filtered through cloth and then through a  $\frac{1}{2}$  inch cotton wad on a Witt filter plate. To the combined filtrates a solution of 150 gm. of neutral lead acetate dissolved in 450 cc. of water was added. After the mixture had stood for 2 days, the lead salt precipitate was removed by decantation and filtration and the clear solution treated with 400 gm. of barium hydroxide. A slimy precipitate formed which required 1 day for removal by filtration. The filtrate was adjusted to pH 1 with sulfuric acid and the barium sulfate was removed by filtration. It was then stirred for half an hour with 100 gm. of charcoal (nuchar) and filtered. The filtrate, which was free from lead, was freed of sulfate ion, concentrated, and fed to Group 25. The charcoal adsorbate was eluted four times with 240 cc. of 50 per cent alcohol adjusted to pH 1 with hydrochloric acid, in each case by being allowed to stand with the eluent several hours before being warmed to 70° and filtered. The combined eluates were partially neutralized, concentrated, and fed to Group 23.

A second extraction was made in a similar manner, except that barium hydroxide solution was added proportionately to but 1 liter of the lead acetate filtrate at a time; the latter was filtered and adjusted to pH 1 with  $\text{H}_2\text{SO}_4$  at once, so that no part of the extract was alkaline for more than 5 minutes. The charcoal eluate obtained from this extract was fed to Group 24.

The lead precipitates from both fractionations were combined, suspended in water, and treated with hydrogen sulfide. The re-

sulting mixture was filtered, and the filtrate was concentrated and fed to Group 22.

All fractions were fed at levels equivalent to 15 per cent of hexane-extracted alfalfa meal. The results are given in Table IV.

The results indicated that the protective factor was lost during the fractionation. The groups receiving charcoal eluates grew more slowly and developed paralysis earlier than the chicks on the basal diet. The factor in alfalfa was apparently largely destroyed by prolonged autoclaving.

TABLE IV

*Results Obtained at 35 Days on Chicks Fed Diet 101 Supplemented with Various Fractions of Hexane-Extracted Alfalfa Meal*

Group No.	Supplement	No. of chicks	No. of survivors	Average weight of survivors	No. of observed cases of paralysis
				gm.	
21	None (basal diet)	13	1	177	8
22	Lead acetate precipitate fraction of water extract	12	1	195	8
23	Eluate from charcoal adsorbate of lead acetate filtrate, Preparation 1	12	0		7
24	Eluate from charcoal adsorbate of lead acetate filtrate, Preparation 2	12	0		6
25	Filtrate from charcoal adsorbate of lead acetate filtrate	12	1	99	8
26	10% hexane-extracted alfalfa, autoclaved at 120° for 5 hrs.	12	5	142	2
27	10% hexane-extracted alfalfa	11	10	192	0

400 cc. of Water Extract 1 were poured into 8 liters of 95 per cent ethyl alcohol. The gummy precipitate was separated from the clear supernatant liquid, which was concentrated to a small volume and taken up with water. Both fractions were fed at a level corresponding to 10 per cent of hexane-extracted alfalfa meal. A water extract was prepared from alfalfa meal in a manner similar to the preparation of Water Extract 1 from hexane-extracted alfalfa meal. The extract frothed during concentration. This was prevented by addition of 20 per cent of ethyl alcohol. This led to



the formation of a precipitate, which was discarded. The filtrate was concentrated to a small volume (Water Extract 2). A group of chicks receiving a supplement of 10 per cent of fat-extracted wheat germ was included, because previous experiments had shown that this material had a marked growth-promoting action when added to a simplified diet. The rice bran extract content of the basal diet was raised to 10 per cent for this series. The results obtained are given in Table V.

Group 28 grew more rapidly and had a lower mortality than Groups 1, 7, 11, 16, or 21. This was attributed to the fact that the rice bran extract content of the basal diet was higher in the

TABLE V

*Results Obtained at 33 Days on Chicks Fed Various Supplements to Diet 101*

Group No.	Supplement (all alfalfa supplements at level equivalent to 10 per cent hexane-extracted alfalfa)	No. of chicks	No. of survivors	Average weight of survivors	No. of observed cases of paralysis
				gm.	
28	None	13	9	152	4
29	Alcohol-soluble fraction of Water Extract 1	12	4	115	3
30	Alcohol-insoluble fraction of Water Extract 1	12	11	236	0
31	Water Extract 1	12	11	218	1
32	Hexane-extracted alfalfa	12	12	247	0
33	Alfalfa Meal 2	11	11	201	0
34	Water Extract 2	12	12	176	0
35	10% fat-extracted wheat germ	12	12	178	0

case of Group 28, and that the rice bran extract supplied a small amount of the protective factor. The data obtained from Groups 28 to 35 are, perhaps for this reason, less clear cut than those of the preceding experiments, but are sufficient to indicate the presence of a protective factor in the supplements fed to Groups 30 to 35. The results obtained with Group 30 indicate that the protective factor is insoluble in 90 per cent ethyl alcohol. The single death in Group 30 occurred on the 3rd day, and hence should probably not be attributed to the diet.

Fat-extracted wheat germ was tested, because it was found previously that this material promoted growth when added to

a simplified diet (16). Subsequent results showed that alfalfa meal also promoted growth when added to this diet.

Other fractions were tested with Diet 101 in a manner similar to that used in the preceding experiments. A water extract of alfalfa meal was prepared so that 4.6 cc. were obtained from 1 gm. of alfalfa meal. The acidity corresponded to a pH value of about 5. 4600 cc. of the extract were shaken with 750 gm. of fullers' earth. The fullers' earth was removed by filtration and washed twice with 500 cc. of water. The filtrate and washings were concentrated to 2100 cc. and treated with 250 gm. of fullers' earth. The filtrate was concentrated under reduced pressure. 500 gm. of the combined adsorbates were eluted with a mixture of water 1000 cc., acetone 400 cc., and ammonia solution 28 cc. The eluate was concentrated under reduced pressure to about 700 cc., neutralized to litmus, and concentrated further. Results of feeding the fractions indicated that the potency of the original extract was divided approximately equally between the eluate and the filtrate. In another experiment, 95 per cent ethyl alcohol was added with stirring to a water extract (3 cc. equivalent to 1 gm. of alfalfa meal) until a concentration of 40 per cent of ethyl alcohol was reached. The precipitate was removed by filtration and washed with 40 per cent ethyl alcohol. The washings were combined with the filtrate and concentrated to a thin syrup which was found to be potent when fed to chicks. The precipitate was inactive.

#### DISCUSSION

A form of nutritional paralysis in chicks was attributed to "vitamin B<sub>4</sub>" deficiency in 1933 by Keenan and coworkers (17). Later papers stated that "vitamin B<sub>4</sub>" was present in peanut oil (18) and hydrogenated cottonseed oil (19). More recently (20) the Wisconsin workers have stated that soy bean oil protects chicks against nutritional encephalomalacia as distinct from "vitamin B<sub>4</sub>" deficiency. "Vitamin B<sub>4</sub>" was originally described as a factor essential for the rat (21). Subsequently doubt was cast on its existence (22). The Oxford laboratory abandoned the method for its assay (23) and György (24) pointed out that the important factor in "Peters' eluate" is the thermostable vitamin B<sub>6</sub> rather than the thermolabile, hypothetical "vitamin B<sub>4</sub>". Elvehjem and Arnold (25) stated that an autoclaved diet known to be low

in thiamine caused occurrence of characteristic vitamin B<sub>1</sub> deficiencies in rats. Administration of thiamine caused an increase in the appetite of rats so that "food consumption increases to a level which supplied sufficient vitamin B<sub>1</sub>." More recently (26) Arnold and Elvehjem state that their autoclaved diet is deficient "in other essential factors" (than thiamine), and have changed to a diet containing added amounts of "factor W." It is evident that the existence of "vitamin B<sub>1</sub>" is not clearly defined. In any case, it appears disadvantageous in the present state of knowledge to apply the same name to an unidentified factor measured with rats and to an unidentified factor preventing paralysis in chicks.

The earlier results in the present investigation harmonized well

TABLE VI

*Summary of Results Obtained on Basal Diet Contrasted with Results Obtained When Supplement of 10 Per cent Alfalfa Meal (in Most Cases Hexane-Extracted) Was Added*

Other experiments showed that the active fraction of alfalfa meal was extracted by warm water.

	Diet 101	Diet 101 + alfalfa meal
No. of groups.....	5	7
Total No. of chicks.....	67	75
Average weight at 14 days, gm.....	64	80
No. of cases of paralysis during experimental period of 33 to 42 days.....	31	0
% mortality during experimental period.....	76	3

with the findings of Goettsch and Pappenheimer (27). The presence of a water-soluble antiparalytic factor in alfalfa meal, however, is not concordant with their results. In the present investigation, it was found that soy bean oil would prevent the paralysis caused by feeding Diet 101 to chicks. Alfalfa meal was more effective in preventing the paralysis. The factor in soy bean oil was previously shown (2) to be present in the ether-soluble, non-saponifiable portion, while the factor in alfalfa meal seems to be not fat-soluble, but water-soluble, and was precipitated by pouring the aqueous solution into 20 volumes of ethanol. It is clear that two factors were encountered, and it is an unusual circumstance that they apparently are independently effective as supplements

to the same basal diet. The growth with soy bean oil; however, was markedly poorer than with active fractions prepared from alfalfa. The results obtained with alfalfa meal are summarized in Table VI, which shows that a definite difference in weight between chicks on the basal diet and chicks receiving an alfalfa supplement had developed by the 14th day, although paralysis was never observed as soon as this in chicks on the basal diet.

Work with other species has indicated the presence of an unknown essential dietary factor in green leaves. Goettsch and Pappenheimer (28) indicate the existence of a factor in green leaves which is necessary to protect guinea pigs and rabbits against a muscular dystrophy which develops when a diet based principally on oats, bran, and dried skim milk is fed. Subsequent work by Morgulis and Spencer (29) led to the conclusion that there must be at least two factors involved in the prevention or cure of muscle dystrophy in rabbits. One factor was present in wheat germ oil, and the other in lettuce or in dry alfalfa. Both factors were present in fresh green alfalfa, or in whole (i.e., unextracted) wheat germ.

Histopathological studies are obviously necessary before it is possible to identify the chick paralysis observed in this investigation. Such studies did not accompany the present investigation, since interest was confined to concentration of the protective factor or factors.

In the cases of Groups 20, 23, 24, and 29, growth was slower and mortality was greater than in the case of chicks on the unsupplemented basal diet. This is possibly a case of antagonistic action between components of a diet. It is perhaps analogous to the observation (9, 12) that the addition of riboflavin to a diet deficient in the filtrate factor accentuates the symptoms of dermatitis in chicks.

#### SUMMARY

1. Further investigations of a nutritional paralysis produced in chicks by feeding a simplified diet (2) have been made. Manganous sulfate was added to the diet to prevent the occurrence of slipped tendons.

2. The addition of alfalfa meal to the diet improved growth and prevented paralysis. Alfalfa meal was more active in these

respects than soy bean oil. The activity of alfalfa meal was largely destroyed by autoclaving for 5 hours at 120°.

3. The active factor in alfalfa meal was not extracted by hexane, but was readily extracted by warm water. The active factor was soluble in 40 per cent ethanol but insoluble in 90 per cent ethanol. It was adsorbed to some extent by fullers' earth from aqueous solution at a pH of about 5, and was eluted from the fullers' earth by a mixture of water, acetone, and ammonia.

4. When the basal diet was supplemented with the portion of an aqueous extract of alfalfa that was soluble in 90 per cent ethanol, or with charcoal eluates prepared from the aqueous extract, or with 0.015 per cent of nicotinic acid amide, greater mortality and slower growth were observed than in the case of chicks on the unsupplemented basal diet. Such results may be due to an antagonistic action between components of the diet.

5. Evidence that there are two different forms of the same active factor or factors was afforded by the observation that protection against paralysis was given by a fat-soluble fraction prepared from soy bean oil (2) or by a water-soluble fraction prepared from fat-extracted alfalfa meal.

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## THE CYSTINE CONTENT OF ACID- AND ALKALI- PREPARED GLUTENIN\*

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The commonly accepted method for the preparation of glutenin, the glutelin of wheat, is that described by Osborne (16). The procedure involves the solution of the protein in 0.2 N NaOH subsequent to the complete removal of all protein material soluble in distilled water, dilute neutral salt solution, and 50 to 70 per cent alcohol. Neutralization of the alkaline solution of the glutenin precipitates the protein which can then be washed and dried with alcohol and ether.

There is a suggestion by Osborne (16) that prolonged treatment with alkali adversely affects the composition of the glutenin, as judged by the lowering of the nitrogen content from 17.34 per cent to 16.70 per cent. Blish and Sandstedt (2) noted the evolution of hydrogen sulfide when extracts of flour in 0.1 N NaOH in 68 per cent alcohol were neutralized with HCl, indicating that cystine or some other sulfur compounds had been altered by the treatment with alcoholic NaOH. The increased sensitivity to alkali of the cystine molecule in peptide linkages has been noted by Bergmann and Stather (1) and also by Brand and Sandberg (4). Hoffman (9) was unable to isolate any cystine from hair that had been washed with a hot 1 per cent solution of  $\text{Na}_2\text{CO}_3$ . Jones and Gersdorff (10), by precipitating casein five times from 0.31 per cent NaOH solution, reduced the cystine content of the casein to less than 10 per cent of its original value as determined by the Sullivan (18) method.

\* The data in this paper are taken, in part, from the dissertation presented by F. J. Neglia in partial fulfillment of the requirements for the degree of Master of Science, Georgetown University, 1938.



As pointed out by Blish and Sandstedt (3) the alkali used in the preparation of glutelins has been of such dilution that serious alteration of the protein molecule has not been suspected. However, Kondo and Hayashi (12) found that the glutelin of rice cannot remain without alteration of its nature when treated with 0.2 per cent alkali. After three or four treatments with this strength of alkali the rice glutelin could no longer be precipitated by neutralization. Blish and Sandstedt (3), using alkali of varying strengths in the initial extraction of the glutenin, prepared proteins differing markedly in their content of amide and basic nitrogen. The stronger the alkali, the less was the amide nitrogen and the more was the basic nitrogen of the treated protein.

Considering these differences in the glutenins thus prepared, Blish and Sandstedt (3) described a method of preparation under conditions which avoid exposure to alkaline reaction at any stage of the procedure. They did not determine the cystine content of either the alkali- or acid-prepared glutenin. Since this amino acid is one very likely to be affected by the alkali method of preparation, a comparison of its content in the proteins prepared by both methods would be of considerable interest and importance.

The cystine content of the glutenin prepared by the alkali method has been determined by various workers. Osborne (16), using an isolation procedure, found 0.02 per cent. Folin and Looney (7) reported 1.80 per cent cystine in glutenin, while Jones, Gersdorff, and Moeller (11), using the Folin-Looney method, found 1.56 per cent cystine. Cross and Swain (5), employing the Van Slyke procedure, found from 0.91 to 1.03 per cent cystine in glutenins prepared from different varieties of wheat. Csonka (6), using the Sullivan (18) method, found from 1.16 to 1.44 per cent cystine in glutenins prepared from hard and soft wheats and from commercial flour. Larmour and Sallans (13), using the Van Slyke procedure, found 0.58 per cent cystine in glutenin prepared according to the customary Osborne procedure and from 1.32 to 1.42 per cent in glutenin prepared by the acid method of Blish and Sandstedt (3). These authors (13) state, "The use of alkaline solutions in preparing glutenin introduces possibilities of wide error in the final analyses, and, therefore, we endorse Blish and Sandstedt's conclusion that it should be strictly avoided."

The cystine values of Larmour and Sallans (13) are the only

ones reported on the glutenins prepared by both alkali and acid dispersions. Since, in the Sullivan (18) method, we have a procedure highly specific for cystine, a study has been made on the cystine and sulfur content of the glutenins made by the method of Osborne and the acid dispersion method of Blish and Sandstedt.

## EXPERIMENTAL

The alkali-dispersed glutenin was prepared according to Blish and Sandstedt's (3) modification of Osborne's procedure, with 0.2 N NaOH. The acid-dispersed glutenin was made according to the method described in the same publication. All the prepara-

TABLE I  
*Ash, Moisture, and Sulfur Content of Glutenins*

Glutenin	Ash	Moisture	Sulfur
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Acid-prepared.....	0.27	6.18	1.32
Alkali-prepared.....	4.30	8.05	1.35

TABLE II  
*Cystine Content of Glutenins, Corrected for Moisture and Ash*

Glutenin	Sullivan	Folin-Marenzi	Okuda
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Acid-prepared.....	1.99	1.98	2.02
Alkali-prepared.....	1.39	1.51	1.46

tions were made from the same sample of commercial wheat flour. Table I gives the percentage of ash, moisture, and sulfur in the two proteins. The sulfur determinations were made by the Parr bomb method. The ash content of the acid-prepared glutenin is considerably lower than that of the alkali-prepared glutenin.

Table II gives the cystine values as determined by the methods of Sullivan (18), Folin-Marenzi (8), and Okuda (15). These values were obtained upon hydrolysates decolorized by carboraffin (carbox E) after a 6 hour hydrolysis of the proteins with 20 per cent HCl, as described by Sullivan and Hess (19). Considerable humin was formed during the hydrolysis of both proteins and,

therefore, recourse was had to other methods of hydrolysis which prevent, to a large degree, the formation of humin. Table III gives the results with the same proteins with the long HCl-HCOOH hydrolysis of Miller and du Vigneaud (14) and a short hydrolysis with HCl-TiCl<sub>3</sub>. The TiCl<sub>3</sub> not only lessens humin formation, as early shown by Sullivan (18), but also, as shown by Sullivan and Hess (20), shortens the time of hydrolysis. In previous work, with purified proteins, Sullivan and Hess found that only cysteine was present in the TiCl<sub>3</sub> hydrolysate. In the 2 hour hydrolysis of the glutenin, however, the colorless hydrolysate left after the removal of the titanium contained cystine predominantly and on short standing only cystine. Accordingly, the determinations were made as for cystine with a cystine standard.

The results by all three methods of hydrolysis (HCl, HCl-TiCl<sub>3</sub>,

TABLE III

*Cystine Content of Glutenins, Corrected for Moisture and Ash*

A = HCl-HCOOH hydrolysis; B = HCl-TiCl<sub>3</sub> hydrolysis.

Glutenin	Sullivan		Folin-Marenzi		Okuda	
	A	B	A	B	A	B
	per cent	per cent	per cent	per cent	per cent	per cent
Acid-prepared .....	2.05	2.09	2.05	2.14	2.15	2.11
Alkali-prepared .....	1.33	1.31	1.52	1.39	1.37	1.37

and HCl-HCOOH) are practically the same in the case of the acid-prepared glutenin and also for the alkali-prepared glutenin, but on a lower level. The Folin-Marenzi procedure tends to give comparatively higher values upon the alkali-prepared glutenin except in the hydrolysis by HCl and TiCl<sub>3</sub>. This difference, however, is probably too small to be of any significance.

All three methods employed for the determination of cystine indicate a marked difference between the two glutenins. Considering the cystine values of the alkali-prepared glutenin, given in Table II, as 100 per cent, the corresponding values of the acid-prepared glutenin are 143, 131, and 138 per cent by the Sullivan, Folin-Marenzi, and Okuda methods, respectively. The other two methods give values substantially identical with that found by the Sullivan method on the alkali-prepared protein. Since these

methods will react with a deaminized or decarboxylated cystine, while the Sullivan method requires the presence of all three groups in the cystine molecule, there is no evidence, from the hydrolysate, that the cystine in the alkali-prepared glutenin has undergone mere deamination or decarboxylation. The evidence seems to indicate, in fact, that there is an actual loss of cystine sulfur in the alkali-prepared glutenin.

The total sulfur values, given in Table I, for the two proteins are practically identical. With the large difference between the cystine content of the hydrolysates of the two proteins the sulfur content of the hydrolysate itself became of importance. By the use of a modified Benedict-Denis method the sulfur content of the two proteins and of 20 per cent HCl hydrolysates of the two proteins was determined. To 250 mg. of the protein or to a hydroly-

TABLE IV

*Sulfur Content of Acid- and Alkali-Prepared Glutenins and Their Hydrolysates, Corrected for Moisture and Ash*

Glutenin	Whole protein	Hydrolysate
	per cent	per cent
Acid-prepared.....	1.28	1.26
Alkali-prepared.....	1.31	1.06

sate of 250 mg. of the protein which had been evaporated to dryness on the water bath, were added 10 cc. of the Benedict-Denis reagent and 100 mg. of  $\text{Na}_2\text{CO}_3$ , as recommended by Rutenber and Andrews (17). The solution was evaporated to dryness on the water bath, 5.0 cc. of concentrated  $\text{HNO}_3$  were added, and the evaporation to dryness repeated. After ignition the regular Benedict-Denis procedure was followed and the sulfur weighed as  $\text{BaSO}_4$ . Table IV shows the sulfur content of the two proteins and of their HCl hydrolysates as estimated by the above modification.

In the case of each of these glutenins the sulfur content of the whole protein as determined by the modified Benedict-Denis method is 97 per cent of the value as determined by the Parr bomb method. The sulfur content of the hydrolysate of the acid-prepared glutenin is 98 per cent of that of the original protein

similarly determined. On the other hand, only 81 per cent of the sulfur of the original alkali-prepared protein is present in the hydrolysate. In the Sullivan method, applied to all three types of hydrolysis (HCl, HCl-HCOOH, and HCl-TiCl<sub>3</sub>), the average difference between the cystine content of the acid- and alkali-prepared glutenin is 0.70 per cent. The difference in the sulfur content of the hydrolysates of the respective glutenins is 0.20 per cent, which is 0.75 per cent calculated as cystine. It would thus appear that the difference between the cystine content of the two proteins can be substantially accounted for by sulfur lost during the hydrolysis of the alkali-prepared glutenin. The alkali treatment, apparently, has so altered a portion of the cystine in the protein molecule that some of its sulfur is labile to acid hydrolysis.

#### SUMMARY

Glutenins have been prepared by both acid and alkali dispersion. The acid-prepared protein contains less ash than the alkali-prepared protein. The sulfur content of both proteins is practically identical. The cystine content of the two glutenins has been determined by the Sullivan, Folin-Marenzi, and Okuda methods upon hydrolysates prepared by 20 per cent HCl, HCl-HCOOH, and HCl, in the presence of TiCl<sub>3</sub>. All the methods upon the various hydrolysates agree in showing that the cystine content of the alkali-prepared glutenin is markedly lower than that of the acid-prepared glutenin.

Sulfur was determined, by a modified Benedict-Denis method, on the hydrolysates of the proteins. While the sulfur content of the hydrolysate of the acid-prepared protein is 98 per cent of that of the whole protein similarly determined, the sulfur content of the hydrolysate of the alkali-prepared protein is only 81 per cent of the sulfur content of the whole protein. The difference between the sulfur values of the two hydrolysates substantially accounts for the difference in the cystine content of the two proteins. It would appear that the acid dispersion method for the isolation of glutenin is to be preferred over the method with 0.2 N NaOH, since in such a preparation the destruction of cystine, a destruction that occurs to a considerable degree even on contact with weak alkali, is avoided.

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## THE CARBOXYLASE ENZYME SYSTEM\*

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Carboxylase was discovered by Neuberg and Hildesheimer in 1911 (1). It is a highly specific enzyme as it only attacks  $\alpha$ -keto acids, such as pyruvic acid, converting them into the corresponding lower aldehydes; e.g., pyruvic acid into acetaldehyde. The coenzyme dependency of carboxylase, however, was only noticed 21 years later by Auhagen (2). This investigator has shown that when dry yeast was washed with  $\text{Na}_2\text{HPO}_4$  it lost its power to decarboxylate pyruvic acid and on the addition of cocarboxylase, which he obtained in a semipure state, the enzyme became active again. To restore full activity, however, magnesium ions appeared to be necessary. This was known to be the case with zymase.

Recently Lohmann and Schuster (3) have isolated cocarboxylase from bottom yeast in a pure state and found that it is the pyrophosphoric acid ester of thiamine. In support of their work it had been shown that dry yeast (4), washed dry yeast (5), and duodenal phosphatase (5, 6) convert thiamine into cocarboxylase. Peters (7) found that "vitamin  $\text{B}_1$  is phosphorylated rapidly under the conditions of a catatorulin test." Stern and Hofer (8) attempted to convert thiamine into its pyrophosphoric acid ester by treatment with phosphorus oxychloride. While this reagent was very useful in the conversion of lactoflavin (vitamin  $\text{B}_2$ ) into flavin monophosphoric ester (9), which is one of the coenzymes of the yellow oxidation system of Warburg and Christian, only a small amount of the thiamine could be phosphorylated by  $\text{POCl}_3$ . Apparently  $\text{POCl}_3$  is not an efficient reagent for the

\* Preliminary reports of part of this paper have appeared (*J. Am. Chem. Soc.*, **60**, 730 (1938); *Proc. Soc. Exp. Biol. and Med.*, **33**, 888, 890 (1938)).



introduction of the pyrophosphate group. Nevertheless, Stern and Hofer have shown by cataphoretic tests that conversion of the vitamin to its diphosphoric ester took place.

In the present paper a synthesis will be described by which thiamine may be completely converted into cocarboxylase. The coenzyme has been obtained in crystalline form. A series of new activators of the carboxylase-cocarboxylase system has been found and a specific function of cocarboxylase has been observed. Experiments will be presented showing that, similar to thiamine, cocarboxylase is also a growth-promoting substance.

#### EXPERIMENTAL

*Synthesis and Purification<sup>1</sup> of Cocarboxylase*—500 mg. of sodium pyrophosphate are placed in a Pyrex test-tube and heated until all of the water of crystallization is removed. 1 cc. of orthophosphoric acid (c.p. 85 per cent) is placed in another large Pyrex test-tube and heated until a slight amount of solid deposit forms on the side of the tube. Then the pyrophosphate is added and the mixture gently heated until solution takes place. After a few minutes of cooling 500 mg. of thiamine are added and the contents of the tube are well mixed. The tube is placed in an oil bath of 155°, kept there for 15 minutes, and constantly stirred. Then the tube is removed and, after cooling, the solid mass is dissolved in 10 cc. of cold water. Cold saturated  $\text{Ba}(\text{OH})_2$  solution is added until no more precipitate forms and the solution is just commencing to turn yellow. (The thiochrome compound of cocarboxylase is inactive. It becomes active again, however, on acidification.) The precipitate is centrifuged off and the supernatant fluid is decanted. The precipitate is extracted three times with 50 cc. of cold water. All four supernatant fluids are united and, after cooling, 3 per cent  $\text{H}_2\text{SO}_4$  is added to slight blue reaction of Congo red paper. The  $\text{BaSO}_4$  is centrifuged off and discarded. The Ba-free solution is concentrated to 30 cc. in a vacuum at 25°. It is cooled in ice water and 15 to 20 volumes of a mixture

<sup>1</sup> In the purification and identification of synthetic cocarboxylase Mr. J. Weijlard of the Research Laboratory of Merck and Company, Inc., has collaborated. While this paper was in press the cocarboxylase was obtained in a 100 per cent pure state. Hydrolysis and cleavage products were analyzed. The results of this work will be published elsewhere.

of 1 part of absolute alcohol and 2 parts of ether is added which precipitates the thiamine pyrophosphate in the form of microscopic needles. Sometimes a gummy mass forms which will turn into long macroscopic needles on short standing in the cold. The product obtained on six recrystallizations from the alcohol-ether mixture, redissolved each time in 10 cc. of 0.1 N HCl and dried in a vacuum over  $\text{H}_2\text{SO}_4$ , is readily soluble in water and free of inorganic salts.

*Activity and General Properties*—This synthetic preparation is practically as active as natural cocarboxylase (very kindly furnished by Professor Lohmann). Phosphorus and thiamine content, however, indicates that my cocarboxylase still contains a small amount of impurities.

Similar to the natural cocarboxylase, the synthetic coenzyme becomes inactive on 15 minutes boiling with N HCl, and the total phosphorus is liberated only with difficulty in about 5 hours at  $100^\circ$ . Kidney phosphatase also hydrolyzes the synthetic coenzyme. In this respect it resembles the cocarboxylase obtained by enzymic synthesis (6).

Cocarboxylase forms the theoretical amount of thiamine when treated by an alkaline ferrieyanide solution. The ferrocyanide formed during the reaction may be converted to Prussian blue and measured colorimetrically (10).

Cocarboxylase (10 micrograms) gives a yellow color with the formaldehyde-azo test (Kinnorsley-Peters (11)). Thiamine gives a red color.

*New Activators of the Carboxylase-Cocarboxylase System*—Recently Lohmann and Schuster (3) have shown that manganese ions activate the carboxylase-cocarboxylase enzyme system better than magnesium ions. Copper, iron, bismuth, zinc, and cadmium salts as well as sodium fluoride inhibit the activity of the enzyme carboxylase (12). It is well known that certain amylases (13) cannot hydrolyze starch in the absence of certain salts. Their activation, however, is not limited to a few ions. One might expect that with carboxylase similar conditions exist. My expectations were correct. Several neutral salts such as  $\text{Na}_2\text{SO}_4$ , NaCl, KCl, as well as NaCN, were found to activate the carboxylase-cocarboxylase system (see Table I). The fact that NaCN activates this system shows that carboxylase is not a heavy metal-

containing enzyme. It activates probably in part because it forms a more reactive enol compound (cyanohydrin) with pyruvic acid. NaCN is a good activator, since only one-tenth the concentration is required to obtain the activation similar to that shown by the neutral salts. While Mn ions activate best, the order of activation obtained by NaCN is close to the one obtained by  $MgCl_2$ .  $Li_2SO_4$  activates slightly and in dilute concentration only.  $NaNO_3$  does not activate. As in the case of amylases, no explanation can be given of why these salts activate the carboxylase-coccarboxylase system.

TABLE I

*Activation of Carboxylase-Coccarboxylase System by Various Salts*

All samples contained 30 micrograms of coccarboxylase except in Experiment 10.

In all experiments described in this paper synthetic coccarboxylase and natural coccarboxylase were employed with identical results.

Experiment No.	Salt employed	Carbon dioxide		
		10 min.	20 min.	30 min.
		c.mm.	c.mm.	c.mm.
1	$MgCl_2$ (0.1 mg. Mg)	53	88	112
2	$MnSO_4$ (10 micrograms Mn)	51	78	98
3	$Na_2SO_4$ (4 mg. Na)	38	64	81
4	$NaCl$ (4 mg. Na)	37	62	79
5	$KCl$ (4 mg. K)	36	62	77
6	$NaCN$ (0.4 mg. Na)	38	63	78
7	$Li_2SO_4$ (0.4 " Li)	25	44	59
8	" (4 mg. Li)	20	35	47
9	No salt	18	34	46
10	" " no coccarboxylase	0	0	0

While it is not difficult to remove all of the coccarboxylase from the dry yeast (Table I, Experiment 10), it is not possible by the extensive washing applied to remove all of the other activators of carboxylase (Table I, Experiment 9), and some activation is apparently caused by the buffer (phosphate). In this experiment acid- and alkali-washed dry yeast was employed (see below).

*Specific Function of Coccarboxylase*—It is believed that coccarboxylase combines with the inactive enzyme carboxylase to form a new compound which in the presence of magnesium ions becomes

highly active. This compound, however, has not yet been isolated. Nor has the enzyme carboxylase been obtained in pure state. In any case nothing is known about the nature or mechanism of "activation" of carboxylase by cocarboxylase. The following experiments will show that one of the functions of cocarboxylase is to protect carboxylase, a very labile enzyme, from destruction.

*Experiment 1*—1 cc. of yeast suspension and 30 micrograms of cocarboxylase in 1 cc. of phosphate of pH 6.2 were placed in the main compartment of a Warburg vessel (17 cc. capacity and two side arms). In one side arm 0.5 cc. of sodium pyruvate (5 mg. of pyruvic acid containing 0.1 mg. of magnesium as  $MgCl_2$ ) was placed. The vessel was connected with a Warburg-Barcroft

TABLE II  
*Showing Protective Function of Cocarboxylase*

Experiment No.	Content of main compartment during first 120 min.	Carbon dioxide (after addition of contents in side arm)		
		10 min.	20 min.	40 min.
		c.mm.	c.mm.	c.mm.
1	Yeast suspension and cocarboxylase	40	66	102
2	" " alone	4	12	24
3	" " and thiamine	5	12	24
4	" " " water	0	0	0

respirometer and shaken for 105 minutes at 30°. Then the stop-cock was closed and after 15 minutes of further shaking the pyruvate was washed in from the side arm.

*Experiment 2*—In another vessel the pyruvate was placed in one side arm and the cocarboxylase was placed in the second side arm, while the main compartment of the vessel contained 1 cc. of yeast suspension. This vessel also was shaken for 120 minutes and then the pyruvate and cocarboxylase were added. Cocarboxylase solutions are very stable between pH 4 and 10 at 30°.

*Experiment 3*—Here 30 micrograms of thiamine (Merck) in 0.1 cc. of phosphate of pH 6.2 and 1 cc. of yeast suspension were placed in the main compartment of the Warburg vessel. The cocarboxylase and pyruvate were added at the end of 120 minutes.

*Experiment 4*—The content of this vessel was similar to that

in Experiment 1. Cocarboxylase, however, was replaced by 1 cc. of  $\text{H}_2\text{O}$ .

It may be seen from Experiment 1, Table II, that when cocarboxylase was immediately added to freshly washed yeast and kept at  $30^\circ$  for 120 minutes in the presence of air  $\text{CO}_2$  formed after the addition of pyruvate very rapidly. If the washed yeast, however, was kept for the same length of time without cocarboxylase, the enzyme carboxylase lost almost all of its activity and there was hardly any  $\text{CO}_2$  formed (Experiment 2). Thiamine had no protective action on carboxylase and no cocarboxylase was formed during the duration of the experiment from the vitamin (Experiment 3). The yeast suspension without added cocarboxylase did not form  $\text{CO}_2$  from sodium pyruvate (Experiment 4). Other experiments (not included in Table I) have shown that the protective function cannot be replaced by Ba-adenosinetriphosphate, Mg-hexosediphosphate, or by reduced glutathione.

In these experiments brewers' yeast which had been washed with alkaline phosphate was employed (see below).

*Removal of Activators and Cocarboxylase from Dry Yeast. Alkaline Washing*—For the cocarboxylase test and for the experiments on the "Specific function of cocarboxylase" brewers' bottom yeast<sup>2</sup> was extensively washed with water and dried at room temperature with the aid of a fan. The alkaline washing was carried out according to Lohmann and Schuster (3). To 2 gm. of dry yeast in a 250 cc. centrifuge flask 100 cc. of 0.1 M  $\text{Na}_2\text{HPO}_4$  at  $30^\circ$  was added and shaken in a shaking machine for 12 minutes. Then the mixture was centrifuged. The supernatant fluid was discarded. This procedure was repeated once more. The material was washed for 3 minutes with 100 cc. of water at  $30^\circ$ . The washed yeast was suspended in 20 cc. of phosphate of pH 6.2 and was so used.

It is important to note that there is no direct proportionality between cocarboxylase concentration (activity) and  $\text{CO}_2$  formation from pyruvate. For instance, if the cocarboxylase concentration is increased 3 times,  $\text{CO}_2$  formation is only doubled after the first 15 minutes of the experiment (3, 6, 8). It should also be noted

<sup>2</sup> I am indebted to the Jacob Ruppert Brewery, through the kindness of Mr. E. Muhlhausen, for furnishing the bottom yeast.

that the carboxylase content of various yeasts differs greatly, and for this reason it is difficult to make exact comparisons of cocarboxylase activities by the enzymic test.

*Acid and Alkaline Washing*—For the salt activation experiments the dry yeast was washed with acid and alkaline phosphate according to Lohmann and Schuster (Table VII (3)). 2 gm. of dry yeast were washed by shaking in a machine for 10 minutes at room temperature, three times with 100 cc. portions of 0.1 M  $\text{KH}_2\text{PO}_4$ , once with 100 cc. of  $\text{H}_2\text{O}$ , twice with 100 cc. portions of 0.1 M  $\text{Na}_2\text{HPO}_4$ , and once with 100 cc. of  $\text{H}_2\text{O}$ . The washed yeast was suspended in 20 cc. of phosphate, pH 6.2. A solution of sodium pyruvate containing 10 mg. of pyruvic acid per cc. was prepared and adjusted to pH 6.2 with NaOH. No mineral acids were introduced. 0.5 cc. of sodium pyruvate (5 mg.) was placed in the side arm of the Warburg vessels. 1 cc. of washed yeast suspension, 0.5 cc. of cocarboxylase, 0.5 cc. of phosphate of pH 6.2, and 0.5 cc. of salt solution were placed in the main compartment; total volume, 3 cc. The temperature was 28°.

*Cocarboxylase Growth Substance*—Thiamine has an accelerating action on the growth of yeast. With cocarboxylase acceleration is more pronounced.

In the presence of a proteose-peptone-sucrose-salt medium 1 microgram of the pyrophosphate per cc. increases the growth considerably in 20 hours at 25°. In the following a set of typical experiments is described. To the medium (pH 5.6) containing 5 gm. of sucrose, 1 gm. of proteose-peptone (Difco), 0.3 gm. of  $\text{KH}_2\text{PO}_4$ , and 0.3 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in 100 cc. of distilled water, 0.1 mg. of thiamine or 0.1 mg. of thiamine pyrophosphate (in 0.2 cc. of water) was added. The medium was made up fresh every day and was boiled for 10 minutes before use. Controls were run without the vitamin or ester. 100 cc. samples of the medium were placed in large culture dishes and to each 1 cc. of a 0.1 per cent suspension of commercial bakers' yeast (Fleischmann) was added. After 20 hours three of each of the samples were united, centrifuged, and weighed. Those with added thiamine pyrophosphate weighed 5.1 gm.; those with thiamine weighed 4.6 gm.; whereas without the vitamin or coenzyme samples weighed 2.7 gm.

## SUMMARY

The synthesis of cocarboxylase (pyrophosphoric acid ester of thiamine) from synthetic thiamine has been described. The synthetic, crystalline coenzyme contains a small amount of impurities. It is, however, as active as the pure natural preparation.

Several salts have been found to be activators of the carboxylase-cocarboxylase system, one of which was NaCN. The fact that NaCN activates the system proves that heavy metals are not a part of this enzyme system. The activation by NaCN is probably due to the formation of a more reactive addition (enol) compound, cyanohydrin, with pyruvic acid.

Experiments have been described which show that cocarboxylase has a specific protective action on carboxylase. The enzyme freed of cocarboxylase is very labile. Cocarboxylase prevents it from rapid inactivation. Adenosinetriphosphate, hexosediphosphate, or reduced glutathione cannot replace cocarboxylase in its protective function.

While in plants thiamine pyrophosphate functions as an indispensable specific coenzyme for carboxylase, in mammalian metabolism it acts as a coenzyme for a pyruvic acid dehydrogenase (14).

Experiments with bakers' yeast show that, similar to thiamine, cocarboxylase also functions as a growth substance, and it appears that the growth-promoting action of thiamine is based on its conversion to cocarboxylase by the living cell. After thiamine has been phosphorylated, it acts as an accelerator of carbohydrate metabolism.

I am grateful to Merck and Company, Inc., and to the Winthrop Chemical Company, Inc., for generous gifts of thiamine, and to Professor Lohmann for a sample of pure (natural) cocarboxylase.

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# DIPHTHERIA TOXIN

## II. THE ACTION OF KETENE AND FORMALDEHYDE

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In the first paper of this series (1), the isolation of a highly toxic protein from *Corynebacterium diphtheriae* filtrates was described. Evidence was presented that this protein was 95 to 98 per cent pure and it was concluded to be identical with diphtheria toxin.<sup>1</sup> The protein was analyzed and characterized, but no prosthetic grouping could be detected which could in any way account for its powerful toxicity. The present communication deals with further experiments designed to locate the toxic groups.

It has been known for a long time that when crude toxic filtrates from *Corynebacterium diphtheriae* are incubated with low concentrations of formaldehyde in alkaline solution at 37°, the toxicity gradually disappears without loss of antigenic properties (Ramon (2), Glenny and Hopkins (3)). It has been generally assumed that some or all of the free amino groups on the toxic molecule are involved in this change, since there is a definite reduction in the number of these groups found on treatment with nitrous acid by the Van Slyke method after detoxication (Hewitt (4), Wadsworth and Pangborn (5), Eaton (6)). Unfortunately, almost all the earlier work was done on crude filtrates or otherwise highly impure preparations. Recently, however, the reaction between highly purified diphtheria toxin and formaldehyde has been studied by Eaton (6) who found about a 30 per cent decrease in Van Slyke amino nitrogen after complete detoxication.

The nature of the reaction between formaldehyde and the simple

<sup>1</sup> In the following pages, diphtheria toxin refers to the pure toxic protein and is not used to denote crude toxic filtrates.

amino acids (to say nothing of its reaction with proteins) is, as yet, but little understood (7). It therefore seemed preferable to use some other reagent more suitable for quantitative investigation. Ketene has been used with success as an acetylating reagent for the free amino and hydroxyl groups of pepsin by Herriott and Northrop (8) and of insulin by Stern and White (9). Moreover, methods for determining the number and kind of groups involved on acetylation of proteins with this reagent have been worked out (Herriott (10)). We therefore decided to investigate the action of ketene on our purest preparations of diphtheria toxin, after having first determined the basic amino acids, histidine, arginine, and lysine. While this work was in progress, several papers appeared by Goldie (11) on the action of ketene on crude toxic filtrates and on toxic filtrates purified by dialysis. Goldie reports that short exposure to ketene destroys almost all the toxicity without affecting the ability of the toxin to flocculate with anti-toxin. After more than 50 per cent of the amino groups had been acetylated, his preparations lost their combining power. Other crude bacterial toxins have been shown by Tamura and Boyd (12) to lose their toxicity after treatment with ketene but as yet no details are available.

#### EXPERIMENTAL

*Preparation of Toxin*—The organisms were grown on the gelatin hydrolysate medium previously described (13) except that pimelic acid, nicotinic acid, and  $\beta$ -alanine were used as accessory growth factors instead of the purified liver fraction (14). 1 per cent maltose was used instead of the 0.3 per cent previously recommended and the Toronto strain of Park-Williams No. 8 was employed instead of the Albany No. 5 culture.

110 liters of crude toxic filtrate containing 42 Lf of toxin per cc. were concentrated *in vacuo* at 25° and purified by fractionation with ammonium sulfate, adsorption of impurities with alumina cream, and by dialysis as described in the previous communication (1). Altogether, the yield was about 6 gm. of nearly colorless product containing 16 per cent nitrogen, 0.00046 mg. of nitrogen per Lf unit, 14,000 M.L.D. per mg., and with a specific rotation of  $-39^\circ$ , thus agreeing closely with former preparations.

*Determination of Basic Amino Acids*<sup>2</sup>—Arginine, histidine, and lysine were determined by isolation, by the method of Block (15). Histidine was isolated and weighed as the nitrilide (Block (16)) instead of as the flavianate. Approximately 1 gm. samples were hydrolyzed with 20 cc. of sulfuric acid (40 cc. of concentrated sulfuric acid plus 140 cc. of water) and used for each determination. The results of the analysis of diphtheria toxin prepared from both Albany No. 5 and Toronto strains of *Corynebacterium diphtheriae* are given in Table I.

*Determination of Amino Nitrogen*—Amino nitrogen was determined with nitrous acid in the Van Slyke apparatus, 20 to 30 mg. of material being used for each analysis, with 15 minutes shaking at high speed. Amino nitrogen was also determined by

TABLE I  
*Basic Amino Acid Content of Diphtheria Toxin*

Source of toxin	Amount taken for analysis (ash-free, dry weight)	Histidine	Arginine	Lysine
	gm.	per cent	per cent	per cent
Toronto SJIII.....	1.000	2.2	3.7	5.3
Albany No. 5 C-1.....	1.132	2.4	3.9	*

\* Owing to an unfortunate accident we were unable to complete the lysine estimation on this sample.

formol titration according to Northrop's method (17). Both these methods checked each other closely and the results were in good agreement with the amino nitrogen found by acetone titration in the previous communication (1). The results by these methods were about double the amino nitrogen value for diphtheria toxin calculated from its lysine content. This point will be discussed later.

*Determination of Apparent Tyrosine Plus Tryptophane Content*—The apparent tyrosine plus tryptophane content before and after acetylation with ketene was determined by the methods of Herriott at pH 8 and 11 (10), except that the color was allowed to develop with the phenol reagent for 2 hours at room temperature

<sup>2</sup> The author is very much indebted to Dr. R. J. Block for allowing him to watch his procedure for the determination of the basic amino acids.

instead of 15 minutes at 38°. The true tyrosine plus tryptophane content of diphtheria toxin was taken as 10.5 per cent (1). The value for the tyrosine plus tryptophane content by Herriott's method was about 47 per cent of this value. The figures given in the fifth column of Table II were calculated from the apparent values, the factor 100/47 being used. Although the color obtained with the phenol reagent and the protein was not a perfect match with that obtained with pure tyrosine, the relative tyrosine contents observed for untreated and acetylated toxin are probably close to correct.

TABLE II  
*Acetylation of Diphtheria Toxin*

Time of acetylation	Amino N			Tyrosine + tryptophane		M.L.D. per mg.	Kf <sub>20</sub> *
	Van Slyke	Acetylated	Formol titration	pH 11 method	pH 8 method		
min.	per cent total N	per cent total N	per cent total N	per cent	per cent		
0	6.1†	0	6.6	10.5	10.5	14,000	15
4	2.6	3.5‡	3.2	10.1	9.4	40	75
10	3.0	3.1	3.1	10.4	8.3	7	200
40	2.6	3.5	3.0	10.1	6.6	<7	>600§

\* Kf is taken to represent the flocculation time in minutes at 42°. The subscript denotes the flocculation titer in Lf units per cc.

† This figure is the average of five determinations.

‡ The reduction in amino nitrogen as calculated from the lysine content is 3.2 per cent.

§ There was no flocculation after 40 hours in the cold and 10 hours at 42°, but the solution was slightly cloudy in the expected zone. After it was blended with a rapidly flocculating toxin, about 30 per cent flocculated in 80 minutes, indicating a 70 per cent loss in combining power.

*Acetylation of Diphtheria Toxin*—Ketene was generated by passing acetone vapor over a hot platinum filament in an apparatus similar to that described by Herriott (18). Approximately 100 mg. of diphtheria toxin in 10 cc. of 2 M sodium acetate solution were placed in a dialyzing bag suspended in 1 liter of 2 M sodium acetate and stirred by an electric stirrer. Ketene was passed through the toxin solutions in the bag for varying lengths of time. The pH inside the dialyzing bag never fell below 6.0 during the course of an experiment. This was essential, since the toxin is unstable

below this pH level (1). At the end of the chosen time limit, the sodium acetate was removed from the bag by dialysis against distilled water and the toxin solution analyzed for total nitrogen, amino nitrogen (by both the Van Slyke method and by formol titration), and apparent tyrosine content by the methods at pH 8 and 11, and the flocculation titer, flocculation time, toxicity, and immunizing power were determined. The results are given in Table II.

*Treatment of Diphtheria Toxin with Formalin*—10 cc. samples of an approximately 1 per cent solution of diphtheria toxin in borate buffer at pH 8.1 were treated with 0.2 per cent and 1 per cent formalin (U.S.P., 37.7 per cent formaldehyde). A third

TABLE III  
*Action of Formalin on Diphtheria Toxin at 38° for 6 Days*

Formalin	pH	Toxin concentration	Amino N, Van Slyke	Amino N, combined	M.L.D. per mg.	Kf <sub>50</sub>
per cent		mg. per cc.	per cent total N	per cent total N		
0	8.1	8.73	6.3	0	14,000	15
0.2	8.1	10.85	2.8	3.5*	0	60
1.0	8.1	10.66	1.9	4.4	0	∞ †
1.0	6.3	11.66	3.3	3.0	0	35

\* Calculated from the lysine content = 3.2 per cent.

† No flocculation. Blending with a rapidly flocculating toxin indicated that more than 70 per cent of the combining power had been destroyed.

10 cc. sample in Sørensen's phosphate buffer at pH 6.3 was treated with 1 per cent formalin. All three samples were incubated for 6 days at 38°. At the end of this time the samples were withdrawn from the incubator, and analyzed in the same way as the acetylated toxin. The results are shown in Table III.

#### DISCUSSION

The only free amino groups known to be determinable in proteins are those due to the  $\epsilon$ -amino groups of lysine. By isolation, the lysine content of diphtheria toxin is 5.3 per cent, corresponding to an amino nitrogen content of 3.2 per cent of the total nitrogen of the protein. However, the average amino nitrogen content of the toxic protein as determined by the Van Slyke method is 6.1

per cent, by the formol titration is 6.3 per cent, and by acetone titration, 7.4 per cent of the total nitrogen. After acetylation of diphtheria toxin by short treatment with ketene, the amino nitrogen immediately falls to 2.75 per cent and remains constant at this figure even after much longer periods of acetylation. At the same time the toxicity of the protein is almost entirely lost. By subtraction, using the figures obtained by the Van Slyke method, we find that 3.35 per cent of the total nitrogen has been acetylated during the detoxication process. It is therefore concluded that the  $\epsilon$ -amino groups of lysine are the groups actually affected during detoxication. At the present time, we are unable to account for the high amino nitrogen content found for the toxin itself or for the residual amino nitrogen remaining after acetylation. It seems improbable that this residual amino nitrogen can be due to a number of terminal  $\alpha$ -amino groups, since both amino groups of lysine and the  $\alpha$ -amino group of arginine were rapidly acetylated by ketene under the identical conditions used for acetylating diphtheria toxin. The possibility that the residual amino nitrogen is due to amide groups split off by nitrous acid in the Van Slyke machine would appear to be untenable, since the same amino nitrogen value is found by formol titration. It seems improbable that the anomaly is due to the presence of traces of ammonia, since we have been unable to remove them by any means.<sup>3</sup> It is at least possible that we are dealing here with some other amino group, not of lysine, which does not react with ketene or with low concentrations of formaldehyde when the protein is in the native state, but is only determinable after denaturation by nitrous acid or by the formol titration. The inability of heme to form a hemochromogen with native egg

<sup>3</sup> 5 cc. of a 3 per cent solution of diphtheria toxin were placed in a thin walled cellophane bag in a 30 cc. test-tube and dialyzed against 60 liters of running 0.1 per cent sodium bicarbonate for 6 days. At the end of this time a determination of the amino nitrogen content (Van Slyke) gave 6.6 per cent of the total nitrogen. The remaining toxin was dialyzed for 2 more days against changes of 0.01 N hydrochloric acid, after which the amino nitrogen had fallen only to 6.25 per cent. Although 80 per cent of the residual amino nitrogen could be steam-distilled from a micro-Kjeldahl apparatus from acetylated toxin in 10 per cent sodium carbonate, less than 20 per cent was found as ammonia after precipitation of the acetylated toxin with trichloroacetic acid.

albumin although it does so with denatured albumin is a case in point (Holden and Freeman (19)).

As shown by Herriott and Northrop (8) and Stern and White (9), the hydroxyl groups of tryosine are acetylated at a slower rate by ketene than the free amino groups. With diphtheria toxin, this is also the case. As increasing numbers of O-acetyl groups are formed, the flocculation time is progressively increased, until finally, when about 35 per cent of the hydroxyl groups has been acetylated, the toxin no longer flocculates with antitoxin, although incomplete combination still takes place when the acetylated toxin is blended with a rapidly flocculating fresh toxin. It has not been possible to regenerate a rapidly flocculating acetylated toxin by hydrolysis of the O-acetyl groups at pH 9 nor has it been possible to immunize guinea pigs against diphtheria intoxication with acetylated toxin. This failure to immunize, however, is probably due to the fact that doses large enough to immunize were lethal to the guinea pigs, except in the case of toxin subjected to such prolonged treatment with ketene that profound alteration had occurred.

The action of low concentrations of formaldehyde on diphtheria toxin at pH 8.1 or of higher concentrations at pH 6.3 appears to be analogous to short treatment with ketene in that there is a reduction in free amino nitrogen corresponding to that calculated from the lysine content. The union with formaldehyde is an irreversible one and therefore cannot be due to the mere formation of methylene linkages to the nitrogen (7). The action of higher concentrations of formaldehyde in alkaline solution results in destruction of antigenic properties, but the reaction is too complex to warrant further discussion at this time.

The finding that acetylation of the  $\epsilon$ -amino groups of lysine causes loss of toxic properties does not mean that these groups are actually toxic in themselves, but are so only by virtue of their spatial arrangement in the toxic protein molecule. This finding does make it seem highly improbable that any prosthetic group analogous to the heme of hemoglobin or the riboflavin of the yellow enzyme will be found in diphtheria toxin. For any further explanation of the enormous toxicity of this protein, it seems probable that a search must be made in the animal tissue itself for some enzyme system specifically affected by the toxin.



## SUMMARY

1. The basic amino acids of diphtheria toxin have been determined. The toxic protein contains 2.3 per cent histidine, 3.8 per cent arginine, and 5.3 per cent lysine.

2. Diphtheria toxin loses its toxicity after short acetylation with ketene at pH 6 to 7 without, however, losing its ability to combine and flocculate with antitoxin. During the detoxication a number of free amino groups are acetylated, corresponding closely to the number of  $\epsilon$ -amino groups of lysine present. An analogous reduction in amino nitrogen is found after detoxication with low concentrations of formalin in alkaline solution.

3. Further treatment of the toxin with ketene results in the acetylation of the hydroxyl groups of tyrosine, causes a lengthening of the flocculation time, and finally destroys its ability to combine with antitoxin.

4. The significance of these findings is discussed.

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# THE OXIDATIVE DEAMINATION OF SOME STRUCTURALLY RELATED AMINOPROPIONIC ACIDS BY THE LIVER AND KIDNEY TISSUES OF THE RAT

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The original observations of Meyerhoff, Lohmann and Meier (1) and of Reinwein (2) on the decomposition of amino acids by surviving tissues have been fully confirmed and extended by a number of investigators (3-5). Krebs (6, 7) has made a particularly thorough study of this problem and has presented excellent evidence that liver and kidney tissues act upon amino acids by a process of oxidative deamination. The results of these studies thus support the older views of Neubauer (8) and Knoop (9).

It is an interesting fact, however, that although the animal organism, as a whole, is concerned chiefly with the metabolism of the naturally occurring amino acids of the *l* series,<sup>1</sup> the individual tissues, in *in-vitro* experiments, exhibit a much greater deaminizing activity toward the unnatural *d*-amino acids. The physiological rôle of the *d*-amino acid deaminase is not clear. Krebs (11) has suggested that this enzyme may be a fragment of the deaminizing system of the intact tissue, in which case the results of the various experiments are of doubtful significance in metabolism. On the other hand, it must be recognized that the *d*-deaminase may function as an agent by means of which the organism resolves racemic mixtures of amino acids and thus produces the *l* forms. There is considerable evidence that the *d* forms of certain amino acids are readily utilized in the animal organism (12-14) and the recent findings of Braunstein and Kritsman (15), that amino acids are synthesized in the tissues by the intermolecular transfer of an amino group from an amino acid

<sup>1</sup> The nomenclature for the optical isomers proposed by Wohl and Freudenberg (10) is used throughout this paper.

to a keto acid, suggest that racemic mixtures of amino acids may arise as intermediates in the metabolic process.

In view of the fundamental importance of these questions to the whole problem of amino acid metabolism, a somewhat sharper definition of the specificity of the deaminizing system is desirable. The experiments reported here are concerned with the relationship of structural isomerism to the specificity of the *d*-deaminase of liver and kidney. A number of aminopropionic acids have been studied with respect to their oxidation and deamination by rat tissues and it has been shown that the enzymes in these tissues act only upon the  $\alpha$ -amino groups of the compounds in this series.

#### EXPERIMENTAL

The accuracy of the experimental technique was checked by a large number of experiments with *dl*-alanine and *dl*-phenylalanine. These amino acids have been the substrates of choice in a number of investigations (3, 5, 16), and the results obtained here are in good agreement with those reported in the literature. It was found, however, that the respiration of the tissues alone was of such a magnitude that the effects of the added amino acids were obscured. Szent-Györgyi (17) has shown that tissue respiration is markedly suppressed by the presence of sodium arsenite which prevents further oxidation of  $\alpha$ -keto acids. Krebs has used this reagent in experiments designed for the isolation of the products of the deamination of amino acids and has shown that its presence has no effect upon the deaminizing system. It has been considered expedient, therefore, to include arsenite in the suspension medium of every experiment. Under these conditions, it was demonstrated that the oxidation of the amino acids mentioned above took place at a fairly rapid rate, and with the kidney tissues the oxygen consumption and the ammonia production approached the theoretical values demanded by the assumption that the *d* isomer alone was oxidized. The respiration of the liver tissues in the presence of the amino acids was not as large as that observed with the kidney tissue nor was the deamination as extensive over a given period of time.

*Amino Acids*—The *d*-, *l*-, and *dl*-alanine and the *dl*-serine were commercial products. The  $\beta$ -alanine was prepared according to the directions of Clarke and Behr (18); *dl*- $\alpha$ ,  $\beta$ -diaminopropionic

acid, by the method of Neuberg and Silberman (19); and *dl*-isoserine, by the ammonification of  $\beta$ -chlorolactic acid (20). All of the amino acids were recrystallized until the presence of ammonia could no longer be detected and until the analyses for total nitrogen and amino nitrogen agreed with the theoretical values.

*Tissues*—The tissues of young male rats, 125 to 150 gm. in weight and previously fasted for 24 hours, were used. The animal was stunned by a sharp blow upon the back of the head and the liver or the kidneys were removed as rapidly as possible and placed in ice-cold Ringer-phosphate solution. The slices were cut with a new razor blade, squared over cross-section paper, and placed in the various manometer vessels. The dry weight of the slices was usually between 5 and 8 mg.

*Solutions*—The buffered medium used throughout the investigation was a Ringer-phosphate solution of pH 7.4, prepared according to the directions of Krebs (6) with the exception that calcium chloride was not included in the mixture.

The stock solution of sodium arsenite was varied according to the volume of the medium to be used in a particular series of experiments. The salt was dissolved in the Ringer-phosphate medium in an amount such that the addition of 0.5 ml. of the solution to the medium in the manometer vessel produced a final concentration of 0.005 M.

The concentration of the amino acid was found to exert a rather marked effect upon the extent of deamination. The optimum concentration varied with the different amino acids. The general procedure adopted was to place in the side arm of the manometer vessel enough of the amino acid solution so that when this was mixed with the tissue and medium the final concentration was either 1 mg. per ml. or 0.005 M. The majority of the studies was made at these concentrations and the agreement in both instances was satisfactory.

*Procedure*—The constant volume Warburg-Barcroft apparatus was used. Seven respirometers were employed in each experiment, one of which served as a thermobarometer. The six experimental vessels thus permitted duplicate determinations with the tissue alone, with the tissue plus the particular amino acid under investigation, and with the tissue plus *dl*-alanine. The study of the latter amino acid was included as a control in every

experiment, since a certain amount of variation in the enzyme content of the tissues of different animals was observed, and, unless the oxygen uptakes with *dl*-alanine were satisfactory, the entire experiment was discarded.

The respirometers were filled with oxygen and were maintained at a temperature of 37°. The experiments were usually continued through a 6 hour period.

At the end of the respiration study, the vessels were opened and the tissues were removed, washed, and placed in tared crucibles to be dried and weighed. The washings were added to the solution in the vessel and this mixture was deproteinized with metaphosphoric acid. Aliquots of the filtrate were used for the ammonia determinations by the Holmes and Watchorn modification of the Sanford method (21).

*Calculations*—All of the respiration data are expressed in terms of the per cent of the theoretical oxygen uptake. These values were calculated in the following manner. The amounts of oxygen consumed by the tissue controls, expressed in cu. microliters, were divided by the dry weights of the respective tissues and the quotients were averaged. The value obtained was the average oxygen consumption per mg. of dry tissue and it has been assumed that this factor was constant for each of the tissue slices. The residual respiration for each of the experimental slices was then calculated by multiplying the dry weight of the tissue slice by the average oxygen uptake of the control tissues. Subtraction of this quantity from the total oxygen consumption gave the oxygen used for the deamination process. This absolute value, expressed in cu. microliters, was then compared directly with the calculated amount of oxygen which would have been required for the complete deamination of that quantity of the amino acid which was present at the beginning of the experiment. The data from the ammonia determinations were similarly treated.

It is believed that this use of absolute percentages, calculated from two types of analytical data, furnishes a superior basis for the comparison of the various experiments than does the expression of the data as rates. This is particularly true in the case of the ammonia in which the values were obtained at the end of the experiment and the rate of formation could not be calculated with any degree of accuracy.

## DISCUSSION

In Tables I and II are presented data typical of the results obtained with a large number of slices of kidney and liver. In these experiments, the concentration of the amino acids was

TABLE I

*Oxidative Deamination by Rat Kidney*

The final concentration of the amino acids was 0.005 M. The theoretical oxygen uptake and ammonia values are calculated upon the basis that one optical isomer only is oxidized. The experiments were of 6 hours duration.

Amino acid	Oxygen consumption			Ammonia production		
	Theoretical	Observed		Theoretical	Observed	
	c.mm.	c.mm.	per cent theoretical	c.mm.	c.mm.	per cent theoretical
<i>DL</i> -Alanine	56	66	118	0.085	0.091	107
	56	58	104	0.085	0.081	95
<i>D</i> -Alanine	112	114	102	0.170	0.162	95
	112	88	79	0.170	0.111	65
<i>DL</i> -Alanine	56	64	114	0.085	0.093	109
<i>L</i> -Alanine	112	-1	-1	0.170	0.001	0
	112	-1	-1	0.170	0.001	0
<i>DL</i> -Alanine	56	63	113	0.085	0.087	102
	56	60	107	0.085	0.085	100
$\beta$ -Alanine	112	-9	-8	0.170	-0.001	-1
	112	-2	-2	0.170	0.002	1
<i>DL</i> -Alanine	56	58	104	0.085	0.082	96
	56	64	113	0.085	0.101	118
<i>DL</i> - $\alpha$ , $\beta$ -Diamino-propionic acid	56	19	34	0.085	0.027	32
	56	22	39	0.085	0.035	41
<i>DL</i> -Alanine	56	72	129	0.085	0.094	110
	56	73	131	0.085	0.099	116
<i>DL</i> -Serine	56	37	65	0.085	0.056	66
	56	38	68	0.085	0.048	56
<i>DL</i> -Alanine	56	46	81	0.085	0.078	92
	56	50	89	0.085	0.079	94
<i>DL</i> -Isoleucine	56	-1	-1	0.085	-0.002	-2
	56	0	0	0.085	0.001	1

0.005 M, but essentially the same results were obtained when the concentrations were 1 mg. per ml. The agreement between the per cent of decomposition as determined by the oxygen consumption and that calculated from the ammonia analysis was quite

satisfactory. In general, the results obtained with the liver slices were more variable and of a lesser magnitude than were those with kidney.

The results with *d*- and *l*-alanine indicate that only the *d* form of this amino acid is attacked. In a number of the experiments with *dl*-alanine, however, values over 100 per cent were obtained, which indicate that the *l* isomer may be decomposed to a small

TABLE II

*Oxidative Deamination by Rat Liver*

The final concentration of the amino acids was 0.005 M. The theoretical oxygen uptake and ammonia values are calculated upon the basis that one optical isomer only is oxidized. The experiments were of 6 hours duration.

Amino acid	Oxygen consumption			Ammonia production		
	Theoretical	Observed		Theoretical	Observed	
	c.mm.	c.mm.	per cent theoretical	c.mm.	c.mm.	per cent theoretical
<i>dl</i> -Alanine	56	22	40	0.085	0.026	31
	56	16	29	0.085	0.022	26
$\beta$ -Alanine	112	0	0	0.170	0.001	1
	112	1	1	0.170	0.002	1
<i>dl</i> -Alanine	56	27	49	0.085	0.046	52
<i>dl</i> - $\alpha$ , $\beta$ -Diamino-propionic acid	56	16	28	0.085	0.022	26
	56	18	32	0.085	0.025	33
<i>dl</i> -Alanine	56	23	42	0.085	0.031	36
	56	19	34	0.085	0.022	26
<i>dl</i> -Serine	56	17	31	0.085	0.018	21
	56	16	28	0.085	0.015	18
<i>dl</i> -Alanine	56	17	31	0.085	0.023	27
	56	16	29	0.085	0.033	39
<i>dl</i> -Isoserine	56	-4	-7	0.085	-0.001	-1
	56	-2	-4	0.085	0.000	0

extent, when present as a component of the racemic mixture. These results are in agreement with those of Krebs who found that certain of the *dl*-amino acids consumed oxygen in excess of the theoretical quantity required for one isomer only.

In no instance could deamination of  $\beta$ -alanine be demonstrated. Krebs has reported similar findings with this amino acid, but Kisch (22) has found that the respiration of horse and ox tissues

was increased by the addition of  $\beta$ -alanine. The maximum value found in the present study was an increase in the respiration of 3 per cent, whereas the average increase was less than 1 per cent. Since these increases are within the limits of error of the methods, it is evident that  $\beta$ -alanine is not decomposed by rat tissues under the conditions of experimentation used here.

Essentially similar results were found with the *dl*-isoserine. In none of the experiments with this amino acid was increased

TABLE III

*Summary of Oxygen Consumption and Ammonia Production*

The concentration of the amino acid was 0.005 M in every experiment. All of the values are expressed in per cent of the theoretical.

Substrate	Tissue	No. of experiments	Oxygen consumption			Ammonia production		
			Maximum	Minimum	Average	Maximum	Minimum	Average
			per cent	per cent	per cent	per cent	per cent	per cent
<i>dl</i> -Alanine	Kidney	22	135	70	106	124	65	100
$\beta$ -Alanine	"	4	2	-8	-2	2	-1	0
<i>dl</i> - $\alpha$ , $\beta$ -Diamino-propionic acid	"	4	39	27	33	41	20	30
<i>dl</i> -Serine	"	4	68	35	53	66	40	53
<i>dl</i> -Isoserine	"	4	0	-3	-2	1	-2	-1
<i>dl</i> -Alanine	Liver	16	63	28	41	54	26	38
$\beta$ -Alanine	"	4	3	-3	0	4	0	3
<i>dl</i> - $\alpha$ , $\beta$ -Diamino-propionic acid	"	4	32	28	30	30	25	28
<i>dl</i> -Serine	"	4	31	10	22	21	16	18
<i>dl</i> -Isoserine	"	4	-1	-7	-5	0	-1	0

oxygen uptake or ammonia production observed. The deaminase of the tissue apparently acts only upon  $\alpha$ -amino groups.

The *dl*- $\alpha$ ,  $\beta$ -diaminopropionic acid was readily attacked by both the liver and kidney tissues but the oxygen uptakes observed were less than one-half of the values which would be expected for the deamination of the  $\alpha$ -amino group of the *d* isomer. Although there is, at present, no direct proof that the deamination observed was the removal of the  $\alpha$ -amino group of this amino acid, the comparison of the results with those obtained with  $\beta$ -alanine and with isoserine indicates that the  $\beta$ -amino group was



probably not attacked. The lower values for the respiration and ammonia production observed with this compound suggest that the introduction of an amino group into the  $\beta$  position of the alanine molecule inhibits, but does not completely abolish the deamination at the  $\alpha$  position. A similar, but less marked effect, was observed with the *dl*-serine when the  $\beta$  substituent is a hydroxyl group. The serine was attacked more readily by kidney tissue than was the diamino derivative, but was less readily decomposed than was the unsubstituted alanine. With liver tissue, the diamino derivative was apparently more easily decomposed than was the serine.

Because of the differences in the deaminizing activity of the slices from the different animals, the data for each amino acid show considerable variation. As has been pointed out, direct comparisons should be made only between the data which were obtained with the tissues from the same animal. As a summary of the various experiments, however, the maximum, minimum, and average per cent of decomposition of each of the amino acids studied is presented in Table III. It is readily seen that the  $\beta$ -alanine and the *dl*-isoserine were not oxidized and that the average values for the *dl*-serine and *dl*- $\alpha,\beta$ -diaminopropionic acid were less than the minimum values found with the control compound, *dl*-alanine.

The authors wish to express their appreciation to Professor Howard B. Lewis for his interest and advice in the conduct of the experiments.

#### SUMMARY

The relationship of the chemical structure of the substrate to the activity of the *d*-amino acid deaminase of rat kidney and liver has been investigated. *dl*-Serine and *dl*- $\alpha,\beta$ -diaminopropionic acid were less readily oxidized than was the control amino acid, *dl*-alanine. *dl*-Isoserine and  $\beta$ -alanine were not attacked. The results indicate that the *d*-amino acid deaminase of rat tissues is a specific enzyme for the oxidative removal of  $\alpha$ -amino groups, and that the substitution of a hydroxyl or an amino group in the  $\beta$  position suppresses but does not completely abolish the oxidative deamination at the  $\alpha$  position.

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# THE COLORIMETRIC DETERMINATION OF SODIUM AS URANYL MANGANESE SODIUM ACETATE

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Since the introduction of the method of Barber and Kolthoff (1) for the gravimetric determination of sodium, and its application to biological materials by Butler and Tuthill (2), a number of colorimetric and microvolumetric modifications have been proposed. These usually involve precipitation of sodium as uranyl zinc sodium acetate, or as the corresponding magnesium salt, with subsequent determination of the uranium in the dissolved precipitate.

Salit (3) used alcohol to effect quantitative precipitation of sodium as the zinc triple acetate, and determined the uranium colorimetrically by conversion to reddish brown uranium ferrocyanide. The method possesses certain disadvantages, chief of which are the instability of the developed color, the somewhat tedious precipitation technique, and the narrow concentration range for color development.

The double uranyl acetates of cobalt (4) and of nickel (5) have been proposed as qualitative reagents for sodium. More recently, Chang and Tseng (6) have studied the use of manganese uranyl acetate, and have found it to compare favorably in sensitivity with the corresponding zinc reagent. Alkaline earth metals and the ammonium radical had little or no effect on this reagent, and considerable quantities of potassium and lithium did not interfere. They assigned the formula  $\text{NaMn}(\text{UO}_2)_3(\text{C}_2\text{H}_3\text{O}_2)_5 \cdot 6\text{H}_2\text{O}$  to the triple acetate.

The present paper deals with the development of a method whereby sodium is precipitated quantitatively as this manganese triple salt. The manganese in the dissolved precipitate is determined colorimetrically by the excellent method of Willard and

Greathouse (7), which involves oxidation of manganous ion to permanganate by means of potassium periodate. The permanganate solutions so prepared are remarkably stable in the presence of a slight excess of periodate, no fading of color being observable over a period of several months.

*Reagents and Special Apparatus—*

1. Uranyl manganese acetate. Solution A consists of 80 gm. of uranyl acetate,  $\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$ , and 46 ml. of 30 per cent acetic acid plus water to make 520 gm. Solution B consists of 245 gm. of manganese acetate,  $\text{Mn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ , and 23 ml. of 30 per cent acetic acid plus water to make 520 gm. Warm on a steam bath until dissolved, mix the two solutions while hot, and allow to cool to room temperature. Add one-third the volume of 95 per cent alcohol, mix in a stoppered flask, and allow to stand for 24 hours before using. If no yellow precipitate appears, add 0.2 gm. of precipitated uranyl manganese sodium acetate in order to saturate with the triple salt. Shake the reagent thoroughly and filter through ashless paper immediately before using.

2. Glacial acetic acid saturated with triple salt, as prepared by Salit. Place approximately 0.1 gm. of triple acetate in a glass-stoppered bottle, moisten with the least possible quantity of water, add 100 ml. of glacial acetic acid (c.p., Baker's Analyzed, Special, 99.6 to 99.7 per cent), and mix the contents. Before using shake the bottle well, allow the precipitate to settle, and filter through ashless paper. There appears to be some tendency for the acetic acid to become desaturated upon standing; hence fresh reagent should be prepared for each day's use.

3. Stock sodium chloride solution. Dissolve 10.1672 gm. of pure dry sodium chloride in water in a 500 ml. volumetric flask, dilute to the mark, and mix well. 1 ml. of this solution contains 8 mg. of sodium.

4. Standard sodium chloride solutions. Prepare three standard solutions by diluting 15, 25, and 45 ml. of the stock sodium chloride solution to 1 liter. 1 ml. of each standard then contains, respectively, 0.12, 0.20, or 0.36 mg. of sodium. The 0.12 mg. standard covers a range of 0.10 to 0.15 mg. of sodium; the 0.20 mg. standard, a range of 0.15 to 0.27 mg.; and the 0.36 mg. standard, a range of 0.27 to 0.50 mg.

5. Potassium periodate reagent. To 5 gm. of potassium perio-

date in a 2 liter flask, add 800 ml. of water and 100 ml. of 85 per cent phosphoric acid. Shake the flask until the periodate has dissolved; then transfer the reagent to a glass-stoppered bottle and keep in the refrigerator when not in use. While no quantitative study of the stability of this solution was made, it was found that reagent stored in the cold is still satisfactory after 3 months. Furthermore, the use of cold reagent prevents premature oxidation of the manganese in the sample.

6. Calcium hydroxide-phenolphthalein mixture. Place 100 gm. of powdered calcium hydroxide and 0.25 gm. of phenolphthalein in a wide mouthed bottle. Stopper and mix well by shaking and rotating the bottle. Keep tightly stoppered when not in use.

7. Powdered mercuric chloride.

8. Hydrochloric acid, concentrated.

9. Ammonium perchlorate, saturated solution.

10. Sulfuric acid, 4 N and 2 N.

11. No. 2 stirring rods as prepared by Salit. Heat a piece of glass tubing and pull out until it is about 1 mm. in diameter. Cut into 15 cm. lengths; then seal and flatten the ends.

12. Apparatus for removing supernatant liquids. Construct a suction tube with U-shaped capillary tip, as described by Peters and Van Slyke (8), and connect it to a 500 ml. filtering flask.

### *General Procedure*

Transfer exactly 1 ml. of the sample solution (containing 0.1 to 0.5 mg. of sodium) to a 15 ml. centrifuge tube, and 1 ml. of each standard sodium chloride solution to three other centrifuge tubes of the same size. To each add 8 ml. of the freshly filtered uranyl manganese acetate reagent. Stir until the precipitate begins to form, and about 1 minute thereafter. Rinse the stirring rod with a few drops of the reagent as it is withdrawn and before transferring it to the next tube. Stopper the tubes and allow them to stand overnight. Remove the stoppers, centrifuge the contents of the tubes for 10 minutes, carefully remove the supernatant liquid by means of the suction apparatus, invert the tubes on a pad of coarse filter paper, and allow to drain for 5 minutes. Wipe the mouths of the tubes with a piece of coarse filter paper; then wash the precipitate in each with 5 ml. of freshly filtered saturated solution of triple acetate in glacial acetic acid. Deliver the reagent

from a pipette by gentle blowing, pressing the pipette against the wall of the tube immediately below its edge while rotating the tube between the fingers. Stir the precipitate thoroughly, distributing it uniformly throughout the wash reagent. Again centrifuge the tubes, remove the supernatant liquid, drain for 5 minutes, and wipe the mouths of the tubes with a piece of moistened filter paper. With a pipette deliver 10 ml. of potassium periodate reagent to each sample in the centrifuge tubes, and stir until the precipitate is completely dissolved. Place the tubes immediately in a boiling water bath and allow them to remain for 10 minutes to effect complete color development. After removing the tubes from the bath, and while the solutions are cooling to room temperature, group the samples with the standards which they most nearly match by placing a sheet of white paper directly behind the tubes and viewing them horizontally against a source of white light. By means of a funnel and a stream of water from a wash bottle, transfer the 0.12 mg. and the 0.20 mg. standards, and also the unknowns which have been grouped with them, to 25 ml. volumetric flasks, dilute to the mark, and mix well. In the same manner transfer the 0.36 mg. standard and the unknowns in this group to 50 ml. volumetric flasks, dilute, and mix. Set the standards at 20 for the final comparison in the colorimeter.

#### *Procedure for Urine*

To a measured volume of urine in a small flask add water to make the volume exactly 10 ml. At the same time transfer 10 ml. of each standard sodium chloride solution to three flasks of the same size. Add 0.2 gm. of the calcium hydroxide-phenolphthalein mixture to each flask; then stopper and mix. The solutions should turn pink. Allow to stand for 30 minutes with occasional mixing; then filter through ashless paper into dry flasks or test-tubes. Transfer exactly 1 ml. of each of the filtrates to 15 ml. centrifuge tubes, and continue as described under "General procedure." If the urine contains protein, add 0.05 gm. of powdered mercuric chloride, mix, and allow to stand for several minutes before adding the calcium hydroxide-phenolphthalein mixture.

For normal urines, dilution of 1 ml. of urine with 9 ml. of water will usually be satisfactory. Urines of low sodium content may be diluted with an equal volume of water, or used undiluted. When

working with urines containing less than 10 mg. of sodium per 100 ml., the urine filtrate must be concentrated and potassium should be removed. Treat 10 ml. of urine with the calcium hydroxide-phenolphthalein mixture as described above. At the same time treat three 10 ml. portions of water in the same manner. Transfer 5 ml. of each of the filtrates to 50 ml. beakers and add concentrated hydrochloric acid, a drop at a time, until the solution is just acid. To each of the three solutions not containing urine add exactly 1 ml. of each standard sodium chloride solution. Evaporate the sample and the standard solutions on the steam bath until the volume has been reduced to about 1 ml. Add 0.75 ml. of saturated ammonium perchlorate solution and let stand for half an hour with occasional mixing while the potassium perchlorate precipitates. Filter into 50 ml. beakers and wash quantitatively with 95 per cent alcohol. Evaporate to about 1 ml. in volume, transfer to 15 ml. centrifuge tubes, and wash the beakers with four successive 2 ml. portions of uranyl manganese acetate reagent, transferring the washings each time to the centrifuge tubes. Stir for several minutes, so that the reagent will dissolve any material which may have crystallized from the solution during the concentration process. Continue as outlined under "General procedure."

#### *Procedure for Blood Serum*

Ash 0.1 ml. of serum by the method of Ball and Sadusk (9), using 0.2 ml. of 4 N sulfuric acid. To the ash add 1 drop of 2 N sulfuric acid and transfer to a 15 ml. centrifuge tube with two 0.5 ml. portions of water. Rinse the crucible with four 2 ml. portions of uranyl manganese acetate reagent, and continue as outlined under "General procedure." For the color comparison use a standard containing 0.36 mg. of sodium.

#### EXPERIMENTAL

In the development of the method a number of experiments were carried out in order to establish the proper conditions for the quantitative precipitation of known amounts of sodium. For this purpose two independent color standards were prepared as follows:

1. A small quantity of pure uranyl manganese sodium acetate was prepared, and 0.5314 gm. of the salt was dissolved in 360 ml.



of water and 40 ml. of 85 per cent phosphoric acid. 2 gm. of potassium periodate were added, and the solution was boiled for several minutes to develop the color, cooled to room temperature, and diluted to exactly 1 liter. 25 ml. of this solution were equal to 0.2 mg. of sodium.

2. To 500 ml. of a solution containing 0.6871 gm. of potassium permanganate were added 15 ml. of 2 N sulfuric acid and 2 gm. of sodium bisulfite. The solution was boiled until colorless and free of sulfur dioxide, cooled, and diluted to 1 liter. This gave a standard solution of manganous sulfate, each ml. of which was equivalent to 0.1 mg. of sodium.

Another solution was prepared by dissolving 5.5329 gm. of uranium acetate in water and diluting to 1 liter. 1 ml. of this solution contained the same amount of uranium as is contained in the triple acetate from 0.1 mg. of sodium.

The color standard was prepared by taking exactly 80 ml. of the manganous sulfate solution and 80 ml. of the uranium acetate solution, adding 40 ml. of 85 per cent phosphoric acid, diluting to 400 ml., and oxidizing with 2 gm. of potassium periodate as in the previous case. After cooling, dilution was made to 1 liter.

The fact that the two standards checked when one was made up on the basis of the formula assigned by Chang and Tseng (6) and the other on the ratio of acetates of uranium and manganese in the triple salt is evidence for the correctness of the Chang and Tseng formula.

The gravimetric zinc uranyl acetate method was used as a check on the results obtained with urine and blood serum. Hald (10) has pointed out the difficulty encountered in keeping the alcohol wash solution saturated with triple acetate, and Salit (3) has suggested that glacial acetic acid saturated with the triple salt is a better wash reagent. During the course of the present work, difficulty was also encountered in the final washing with ether. Because of the high relative humidity which prevails much of the time in this locality, there was a tendency for the rapidly evaporating ether to cause condensation of water on the inside of the filtering crucible, thus rendering possible the loss of precipitate by solution. In the attempt to substitute for ether a liquid of higher boiling point, it was found that ethyl acetate was highly satisfactory, since it is completely miscible with glacial acetic acid and

exerts very little solvent action on the triple acetate. Precipitates were washed with five 2 ml. portions of the Salit wash reagent, followed by two 5 ml. portions of ethyl acetate. The suction was shut off during the addition of each portion of reagent, so that the precipitate was completely covered by the liquid. After the final washing the crucible was sucked dry for 2 minutes, carefully wiped on the outside, and transferred to the desiccator.

### Results

Table I shows the results of determinations on known amounts of sodium chloride and on synthetic urine. The independent color

TABLE I  
*Recovery of Known Amounts of Sodium*

Sample	Volume of sample	Na taken	No. of determinations	Na recovered
	ml.	mg.		mg.
NaCl solution	1	0.100	6	0.100 $\pm$ 0.001
" "	1	0.500	6	0.501 $\pm$ 0.003
Synthetic urine*	1	0.100	3	0.101 $\pm$ 0.001
" "	5†	0.100	2	0.104 $\pm$ 0.001
" "	5†	0.120	2	0.121 $\pm$ 0.002

\* The composition of the synthetic urine, in mm per 100 ml., was as follows:  $\text{KH}_2\text{PO}_4$ , 3;  $\text{K}_2\text{SO}_4$ , 1.2;  $\text{CaCl}_2$ , 0.6;  $\text{MgSO}_4$ , 0.6;  $(\text{NH}_4)_2\text{SO}_4$ , 0.8;  $\text{NH}_4\text{Cl}$ , 4.3. The above solution was used for the blank determinations. Sodium chloride was added in preparing the sample solutions. Calcium hydroxide-phenolphthalein mixture was used to remove phosphate.

† Samples were concentrated and potassium was removed by the procedure given for urines very low in sodium.

standards were used more or less interchangeably throughout this work. Solutions from the samples containing 0.5 mg. of sodium were diluted to 50 ml. for the final comparison against undiluted standard. Solutions from the samples containing 0.10 and 0.12 mg. of sodium were diluted to 25 ml. and compared with a standard prepared by diluting 15 ml. of the independent color standard to 25 ml., giving a standard equal to 0.12 mg. of sodium per 25 ml. Blanks were determined by color comparison in Nessler tubes against portions of the independent standard which had been appropriately diluted.

Tables II and III show the results obtained by the method in the analysis of several samples of urine and one sample of dog serum, as compared with the gravimetric method of Butler and Tuthill (2). Independent color standards were used in the earlier part of the work on urines, but because of the precaution necessary in order to prevent loss of precipitate from the blanks, it was

TABLE II  
*Determination of Sodium in Urine*

Urine No.	Colorimetric	Gravimetric	Urine No.	Colorimetric	Gravimetric
	<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>		<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>
1*	402	398	7	566	566
2*	312	313	8†	554	552
3*	221	216	9	730	727
4*	482	483	10	114	114
5	456	453	11†	2.9	3.0
6	71.0	72.4			

\* Independent color standard used.

† Protein removed with mercuric chloride.

TABLE III  
*Determination of Sodium in Blood Serum*

Colorimetric	Gravimetric
<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>
341	340
338	339
338	339
336	340
343	340
Average..... 339	340

deemed more practical to carry known amounts of sodium through the procedure, and to use these as the standards. This method also eliminates the necessity of using Nessler tubes in the determination of the blanks.

#### DISCUSSION

When 8 ml. of manganese uranyl acetate reagent were added to 1 ml. of water, there was no observable precipitate after overnight standing. The blank obtained (about 0.002 mg.) must be attrib-

uted to the small amount of triple acetate dissolved in the wash reagent which adheres to the inner wall of the tube. In the case of water treated with the calcium hydroxide-phenolphthalein mixture, a visible precipitate was obtained, owing to traces of sodium in the calcium hydroxide. The blank in this case was about 0.003 to 0.004 mg. higher than that obtained on water alone.

One should not expect phosphate to interfere, even though it is precipitated as uranyl phosphate, because by this method manganese rather than uranium is determined. However, high results are obtained unless phosphate is removed by means of the calcium hydroxide-phenolphthalein mixture. A possible explanation is that the uranyl phosphate carries down some of the manganese from the reagent. Removal of phosphate from blood serum is not necessary, since the amount is so small as compared with the sodium that the error introduced is negligible.

#### SUMMARY

A method is presented whereby 0.1 to 0.5 mg. of sodium from urine or blood serum is precipitated quantitatively as uranyl manganese sodium acetate. The manganese in the dissolved precipitate is oxidized to permanganate with potassium periodate, and compared with an appropriate standard.

The author wishes to express his thanks to Dr. B. M. Hendrix, of this Laboratory, and to Dr. H. L. Lochte, of the Department of Chemistry, University of Texas, Austin, for helpful suggestions and criticisms during the course of this work.

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## DERIVATIVES OF *d*-GALACTURONIC ACID

### IV. THE PREPARATION OF THE METHYL ESTER OF *d*-GALACTURONIC ACID\*

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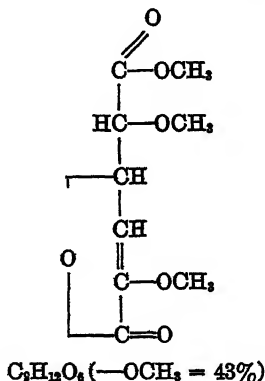
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The synthesis of the methyl ester of *d*-galacturonic acid was accomplished practically simultaneously by Morell and Link (1) and Reichstein and Grüssner (2). Diazomethane in ethyl ether was used as the esterifying agent. Morell and Link used as the starting product the dehydrated  $\alpha$ -*d*-galacturonic acid, while Reichstein and Grüssner started with  $\beta$ -*d*-galacturonic acid which crystallizes in the anhydrous form. Although the details of the procedures employed by each group of investigators were not identical, the ester crystallized out in the  $\alpha$  form,  $[\alpha]_D^{25} = +75.5^\circ$ , and had a melting point of 146–148°. Morell and Baur were able to make the methyl ester of *d*-galacturonic acid several times by the procedure developed in this laboratory (3). However, subsequent collaborators found it difficult to obtain crystalline preparations by either the Morell and Link procedure or the one published by Reichstein and Grüssner. The same difficulty was experienced by others who were kind enough to inform one of us (K. P. L.).

Methyl- $\alpha$ -*d*-galacturonate is a valuable intermediate for any study that has as its objective the synthesis of acylated derivatives of *d*-galacturonic acid (1) or the  $\alpha$ -acetobromo-*d*-galacturonic acid methyl ester (3–5). Consequently, we have made a careful study of the precise conditions that must be maintained when the carboxyl group of *d*-galacturonic acid is to be methylated selectively with diazomethane.

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

While this study was in progress, Schmidt and coworkers (6) published some results of their investigations on the methylation of dicarboxylic acids with diazomethane. They showed that when *d*-saccharic acid is treated with diazomethane under conditions that do not restrict the methylation to the carboxyl groups (esterification) deep seated changes can take place. In addition to normal esterification, there may occur ether and lactone formation with the simultaneous formation of an unsaturated carbon to carbon bond. Schmidt and coworkers (6) isolated a product with a molecular weight of 216, a melting point of 87°, and  $[\alpha]_D = +84^\circ$  that contained three methoxyl groups for 6 carbon atoms for which they advanced the structure indicated.



The work of Morgan (7) on the esterification of the specific soluble polysaccharide substance of pneumococcus Type I with an ethereal solution of diazomethane showed that ether formation can also take place. Our colleague, Mr. H. A. Campbell (unpublished work)<sup>1</sup> showed that when *d*-galacturonic acid is treated with an excess of diazomethane (*i.e.*, more than the amount required for ester formation) the resulting uncrystallizable syrup<sup>2</sup> could not be used for the synthesis of the mercaptal methyl ester (8). No mercaptal residues could be introduced. This indicates

<sup>1</sup> Part of a thesis to be submitted to the Graduate Faculty of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>2</sup> The same holds for the uncrystallizable syrups obtained from either the Morell and Link (1) or the Reichstein and Grüssner (2) procedure.

that if the esterification of the carboxyl group is not performed with great care glycoside formation takes place, preventing subsequent mercaptalation.

The work of Schmidt and coworkers (6), Morgan (7), and Campbell and Link (8) offers the clue to the failures that have been encountered in the synthesis of the methyl ester of *d*-galacturonic acid. If the proper precautions are not exercised in the esterification of the carboxyl group, competitive reactions take place which lead to non-crystallizable syrups. If one adheres rigidly to the procedure described below, the preparation of methyl-*d*-galacturonate, as well as methyl-*d*-glucuronate (9), offers no difficulties.

#### EXPERIMENTAL

*Preparation of Methyl-d-Galacturonate*—We recommend that the diazomethane be prepared from N-nitrosomethylurea according to the directions given by Arndt (10) and that precautions be taken to have the preparation pure. If it is to be stored, it should be in a brown bottle in a refrigerator at 4°.

Due to the extremely poisonous properties of diazomethane and the explosive tendencies, we strongly advise that not more than a total of 10 gm. of N-nitrosomethylurea be decomposed at one time. In the decomposition of the 10 gm. of N-nitrosomethylurea with potassium hydroxide, small quantities (1 to 2 gm.) of the former should be added to the aqueous potassium hydroxide-ether mixture under thorough shaking at 0°. The ether employed must be peroxide-free.

We also feel obligated to call attention to the following precautionary measures. The operator should wear armor-plate glasses, and all work with diazomethane should be carried out under an efficient hood, preferably one equipped with shatter-proof windows. The operator is urged not to wear shoes shod with soles that lead to the accumulation of static charges (rubber compositions or rubber crepe). A water-driven turbine should be employed to propel the stirrer of the apparatus used in methylating with diazomethane.

25 gm. of  $\alpha$ -*d*(+)-galacturonic acid (m.p. 160°,  $[\alpha]_{589.3}^{25} = +65^\circ$ , dried over phosphorus pentoxide at 70° for 5 hours at 5 mm.) are dissolved in 400 cc. of absolute methanol at room tempera-



ture. Any insoluble or flocculent material should be removed by filtering the solution through a dry Jena sintered glass funnel into a dry flask. The methanol solution is then transferred to a dry, 3 liter, 3-necked flask equipped with a dropping funnel, stirrer (mercury seal, etc.), thermometer, and calcium chloride drying tube. The flask and contents are immersed in an ice bath at  $-10^{\circ}$  and throughout the course of the reaction the temperature of the solution should not rise above  $0^{\circ}$ .

While the reaction mixture is being stirred vigorously, an ethereal solution of diazomethane which has been carefully dried over potassium hydroxide (pearls, free from dust) and which is at  $5^{\circ}$  or below, is added dropwise. From 60 to 70 gm. of *N*-nitrosomethylurea, depending on the extent to which loss of the diazomethane is prevented, are usually required for complete esterification.

To ascertain whether the reaction is complete, the mixture is tested with moist Congo red paper and diazomethane is added until a neutral reaction is observed. After neutrality to Congo red paper is reached, diazomethane is added gradually until there is a slightly acid reaction to moist blue litmus paper.

Thereupon the reaction mixture is stirred for about 10 minutes and filtered from suspended flocculent material. The ether and methanol are completely removed under reduced pressure (7 to 10 mm.) at  $50^{\circ}$ . The resulting syrup is dissolved in 40 cc. of hot, redistilled dioxane. The solution is kept at room temperature for several hours and then at  $5^{\circ}$  or below for 24 hours. Crystallization will usually proceed spontaneously. The crystals are collected on a Buchner funnel and washed with 75 cc. of an ether-dioxane mixture (equal parts) and finally with 50 cc. of dry ether.

An additional crop of crystals can be obtained by concentrating the mother liquors to a volume of approximately 20 cc. Crystallization from the mother liquors usually proceeds rather slowly (2 to 3 days).

The product should be dried at room temperature over concentrated sulfuric acid and calcium chloride for 24 hours at 8 to 10 mm. pressure. Approximately 20 gm. of the ester with a melting point of  $105-110^{\circ}$  (this may vary with different preparations) are usually obtained. The product can be used directly for acylation purposes with good results (1, 3).

The pure methyl ester with a melting point of 146-148° and with an equilibrium value of  $[\alpha]_{589.3}^{25} = +38^\circ$  can be obtained<sup>3</sup> by recrystallizing the crude product from a mixture of 10 parts of dioxane and 5 parts of absolute methanol.

The above esterification procedure can also be used for the preparation in good yields of methyl-*d*-glucuronate which was originally prepared by Goebel and Babers by allowing methyl iodide to act upon silver glucuronate (9).

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<sup>3</sup> As a rule the product obtained by the above method will show an initial rotation approximating +75°. Values between 75° and the final equilibrium value may be observed.



## DERIVATIVES OF *d*-GALACTURONIC ACID

### V. THE SYNTHESIS OF THE METHYL ESTERS OF CHOLESTEROL, SITOSTEROL, AND ERGOSTEROL TRIACETYL-*d*-GALACTURONIDES\*

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(Received for publication, June 3, 1938)

A few sterol glycosides are known but to date no work on sterol glycuronides has been reported (1-4). The work of Marrian and coworkers (5-7) and Venning and Browne (8) has shown that certain sex hormones occur in the urine of mammals as conjugated hexuronides—the so called *gepaarte Uronsäure* (9). It is possible that sterol glycuronides occur in animal and in plant tissues.

It appears that the only work reported so far in which a hexuronic acid has been coupled to a complex containing the phenanthrene nucleus is that of Cook and DeWorms (10) who attempted to condense 4'-hydroxy-3,4-benzpyrene with bromo-2,3,4-triacetyl methyl-*d*-galacturonate first prepared by Morell, Baur, and Link (11). A preliminary report on the product of this reaction indicates that coupling had occurred but difficulties were encountered in isolating the desired complex in the crystalline state. The amorphous product obtained was not analytically pure.

In this communication we describe the successful coupling of bromotriacetyl methyl-*d*-galacturonate (11) with three substances containing the phenanthrene nucleus; namely, cholesterol, sitosterol, and ergosterol. Condensation of the galacturonate with the sterols was effected by employing essentially the conditions used by Fischer and Helferich for glycoside synthesis (12).

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The bromoacetomethyl-*d*-galacturonate was coupled with the sterols in benzene solution, with silver carbonate as the condensing agent. Attempts made to isolate the free sterol galacturonides led to the formation of amorphous powders which could not be easily purified and characterized.

In this respect the sterol galacturonides resemble the sterol glucosides. The most extensive work on the latter is that of Lettre and Hagedorn (13) who showed that the sterol glucosides can be characterized and purified most satisfactorily over the acetyl derivatives. In agreement with the significant findings of Lettre and Hagedorn we found that cholesterol, ergosterol, and sitosterol triacetyl-*d*-galacturonide methyl esters could be readily obtained in a crystalline and analytically pure condition.

The procedure adopted in this work was therefore to remove the unused sterol from the reaction mixture by direct crystallization and through the use of digitonin. The sterol triacetyl-*d*-galacturonide methyl esters could then be crystallized directly.

It is a well recognized fact that *glycosides* are usually much more soluble in aqueous solutions than their corresponding *aglycones*. This generalization holds for the saponins (14) and related cardiac glycosides (15), the tannins (16-18), and the anthocyanidins (19), as well as other glycosides (20). It is also well known that cancer-producing hydrocarbons containing the phenanthrene nucleus have an exceedingly low solubility in water.

Cook and DeWorms (10) have sought to produce water-soluble cancer-producing compounds which might permit the production of cancer at other sites than that of the application of the carcinogenic substance. To gain this end they combined 4'-hydroxy-3,4-benzpyrene with bromoacetylglucose. The 4'-hydroxy-*d*-glucoside of 3,4-benzpyrene was not water-soluble. Consequently, they attempted to prepare the corresponding 4'-hydroxy-*d*-galacturonide of 3,4-benzpyrene with the hope that the carboxyl group of the galacturonide residue, with its salt-forming capacity, might render the complex more water-soluble. A final report on the success of this attempt is awaited with interest.

The methods employed below indicate how pure *substituted* sterol galacturonides might be synthesized. Unfortunately our experience indicates that the water solubility of the free sterol

galacturonides is of a very low order and it appears unlikely that substances containing the phenanthrene nucleus can be rendered water-soluble by a single hexuronide residue. We intend to explore the possibilities of enhancing the water solubility of certain sterols by combining them with strongly hydrophilic polygalacturonide intermediates,  $(C_6H_8O_6)_{8-10}$ , that we have available from other phases of our hexuronic acid studies (21).

#### EXPERIMENTAL

All analyses reported were made by the Pregl methods. The rotations reported were determined with a Franz Schmidt and Haensch polarimeter No. 52b with monochromatic light. The melting points were made in a Thiele tube. The temperature was elevated at the rate of about  $2^\circ$  per minute in the determinations.

*Preparation of Cholesterol Triacetyl- $\beta$ -d-Galacturonide Methyl Ester*—To 4 gm. of cholesterol with a melting point of  $148.5^\circ$  and  $[\alpha]_{589.3}^{23} = -39.2^\circ$  dissolved in 100 cc. of anhydrous benzene, were added 4 gm. of silver carbonate and 4 gm. of  $\alpha$ -acetobromo-d-galacturonic acid methyl ester (11) of a melting point of  $129^\circ$  and  $[\alpha]_{589.3}^{25} = +247^\circ$ . The mixture was shaken until the solution gave a negative halide test. After filtration, the solution was evaporated *in vacuo* to dryness. A yield of 2.5 gm. of unused cholesterol, m.p.  $148.5^\circ$ , was obtained by recrystallizing the residue from 50 cc. of absolute methanol. The filtrate was concentrated to dryness *in vacuo*. It was taken up in 20 cc. of hot methanol and upon standing yielded 1.4 gm. of an amorphous powder having an indefinite melting point. The last traces of sterol were removed by dissolving the powder (1.4 gm.) in 15 cc. of chloroform and precipitating the cholesterol by adding 50 cc. of a 1 per cent solution of digitonin in ethanol. The sterol-digitonide was filtered on a Buchner funnel and washed with ether. The filtrate was concentrated to dryness and then dissolved in 25 cc. of hot methanol. When the solution was slowly cooled, the derivative crystallized out in long needles. Yield 1.0 gm., or 14.1 per cent of the theoretical. It was recrystallized from methanol until a constant melting point and rotation were obtained. The following properties were observed.

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*Melting Point*—219–220°

*Rotation*— $[\alpha]_{589.3}^{27.5} = -6.36^\circ$  in U.S.P. chloroform, where  $c = 1.0$  per cent

*Solubility*—Soluble in chloroform, ether, benzene, hot methanol, hot ethanol, dioxane, acetone, and toluene, and insoluble in water

*Analysis*— $C_{32}H_{50}O_8(COCH_3)_3COOCH_3$

Calculated. C 68.3, H 8.9,  $OCH_3$  4.41

Found. " 68.6, " 9.1, " 4.38

*Preparation of Ergosterol Triacetyl- $\beta$ -*d*-Galacturonide Methyl Ester*—To 4 gm. of ergosterol, m.p. 164° and  $[\alpha]_{589.3}^{25} = -131.7^\circ$ , dissolved in 100 cc. of anhydrous benzene, were added 4 gm. of silver carbonate and 4 gm. of  $\alpha$ -acetobromo-*d*-galacturonic acid methyl ester (11), m.p. 129° and  $[\alpha]_{589.3}^{25} = +247^\circ$ .

The mixture was shaken until the solution gave a negative halide test. After filtration, the solution was concentrated to dryness *in vacuo*. The residue was dissolved in 150 cc. of hot 2:1 alcohol-benzene solution and, when the solution was cooled, 2.1 gm. of ergosterol, m.p. 163°, crystallized. The filtrate was concentrated to 20 cc. and 2.1 gm. of an amorphous powder of indefinite melting point were obtained. An additional quantity of unused sterol (0.4 gm. of ergosterol) was removed by dissolving the amorphous powder in 10 cc. of benzene and permitting it to crystallize at room temperature. The last traces of sterol were removed by dissolving the amorphous powder (1 gm.) in 15 cc. of chloroform and precipitating the ergosterol by adding 100 cc. of a 1 per cent solution of digitonin in ethanol. The sterol digitonide was filtered on a Buchner funnel and washed with ether and the filtrate evaporated to dryness *in vacuo*. The product was taken up in 50 cc. of hot ethanol and, as the solution was gradually cooled, the product crystallized out in long needles. Yield 1.0 gm., or 13.9 per cent of the theoretical. It was recrystallized from ethanol until a constant melting point and rotation were obtained. The following properties were observed.

*Melting Point*—204–205°

*Rotation*— $[\alpha]_{589.3}^{25} = -27.9^\circ$  in U.S.P. chloroform, where  $c = 1.0$  per cent.

*Solubility*—Soluble in chloroform, ether, benzene, hot methanol, hot ethanol, dioxane, acetone, and toluene, and insoluble in water

*Analysis*— $C_{32}H_{48}O_8(COCH_3)_3COOCH_3$

Calculated. C 69.1, H 8.5,  $OCH_3$  4.35

Found. " 69.2, " 8.5, " 4.22

*Preparation of Sitosterol Triacetyl- $\beta$ -D-Galacturonide Methyl Ester*—To 4 gm. of sitosterol with a melting point of  $138^{\circ}$  and  $[\alpha]_{589.3}^{25} = -34.8^{\circ}$ , dissolved in 100 cc. of anhydrous benzene, were added 4 gm. of silver carbonate and 4 gm. of  $\alpha$ -acetobromo-*D*-galacturonic acid methyl ester (11), m.p.  $129^{\circ}$  and  $[\alpha]_{589.3}^{25} = +246^{\circ}$ . The mixture was shaken until the solution gave a negative halide test. The solution was filtered and concentrated to dryness *in vacuo*. The product was dissolved in 50 cc. of hot acetone and permitted to crystallize at room temperature. The crystals (1.4 gm.) were unused sterol, which on recrystallization from methanol gave the accepted melting point for sitosterol. The filtrates were concentrated to dryness and taken up in 25 cc. of hot methanol and permitted to crystallize. The resulting precipitate (2.8 gm.) was filtered off and the last traces of sterol were removed from the amorphous powder by dissolving in 15 cc. of chloroform and precipitating the sitosterol by adding 200 cc. of 1 per cent solution of digitonin in ethanol. The sterol digitonide was filtered off, washed with ether, and the filtrate evaporated to dryness *in vacuo*. The residue was taken up in 25 cc. of hot absolute methanol and, as the solution was gradually cooled, the derivative crystallized out in small needles. Yield 1.3 gm., or 17.7 per cent of the theoretical. The product was recrystallized from methanol until a constant melting point and rotation were obtained. The following properties were observed.

*Melting Point*— $172-173^{\circ}$

*Rotation*— $[\alpha]_{589.3}^{25} = +1.0^{\circ}$  in U.S.P. chloroform, where  $c = 1.0$  per cent.

*Solubility*—Soluble in chloroform, ether, benzene, hot methanol, hot ethanol, dioxane, acetone, and toluene, and insoluble in water

*Analysis*— $C_{54}H_{84}O_8(COCH_3)_3COOCH_3$

Calculated. C 69.3, H 8.6,  $OCH_3$  4.27

Found. " 69.2, " 8.9, " 4.19

#### SUMMARY

1. The cholesterol, ergosterol, and sitosterol triacetyl- $\beta$ -*D*-galacturonide methyl esters have been synthesized by combining the corresponding sterols with  $\alpha$ -acetobromomethyl-*D*-galacturonate.

2. The free sterol galacturonides are amorphous, water-insoluble powders that cannot be characterized or purified with



ease. However, the acetylated sterol galacturonide methyl esters can be purified readily and isolated in the crystalline state.

Our thanks are due to Mr. H. A. Campbell for making the carbon and hydrogen microanalyses. We are indebted to Dr. R. E. Kremers, Research Department, General Foods Corporation, Battle Creek, Michigan, for the sitosterol used in this study.

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## THE CHEMISTRY OF LIGNIN

### XI. LIGNIN FROM WHEAT STRAW\*

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(Received for publication, June 13, 1938)

In the previous communications of this series (1-6), results were presented on the chemistry of the lignins from corn-cobs, oat hulls, and barley and oat straws. In this paper results of a study are given on the chemistry of some of the lignin fractions isolated from wheat straw.

Marion (7) treated wheat straw with methyl cellosolve (mono-methyl ether of ethylene glycol) in the presence of hydrochloric acid, and obtained a product which contained methyl cellosolve in chemical combination with the lignin. By subjecting this product to successive extractions with organic solvents, it was possible to resolve it into several fractions, none of which, however, was homogeneous.

Following, in general, the procedure previously described (5, 6), we isolated three lignin fractions from wheat straw. The first two fractions were isolated from the straw by extracting it successively and exhaustively, first with an alcoholic sodium hydroxide solution at room temperature, and then by refluxing with 4 per cent aqueous sodium hydroxide solution. The third lignin fraction was isolated from the residual straw by the fuming hydrochloric acid method. The composition of the lignin fraction isolated by means of alcoholic sodium hydroxide solution agreed with that represented by the formula  $C_{42}H_{48}O_{16}$ . The alkoxy groups in this lignin fraction were definitely shown to be methoxyls, and approximately four of these groups and five hydroxyl groups capable of being acetylated were shown to be present. Three of the hydroxyl groups could be methylated with diazomethane, thus

\* Industrial Farm Products Research Division Contribution No. 288.

indicating their more acidic or phenolic character. When this lignin fraction was fused with potassium hydroxide, protocatechuic acid was obtained. The yield was 4.5 per cent of the weight of the lignin.

The analytical results on the composition of the lignin fraction isolated by means of the aqueous sodium hydroxide solution are in agreement with that represented by the empirical formula  $C_{40}H_{42}O_{18}$ . Approximately four hydroxyl and four methoxyl groups were shown to be present.

The lignin fraction isolated from the residual straw by the fuming hydrochloric acid method contained substantially the same percentage of methoxyl as that found in the lignin fraction isolated by means of the alcoholic sodium hydroxide solution. This lignin fraction was found to contain a higher percentage of carbon than either of the other two lignin fractions.

#### EXPERIMENTAL

The wheat straw was first exhaustively extracted with a 1:2 alcohol-benzene solution and 1000 gm. of this material (922 gm. of moisture-free material) were repeatedly extracted with alcoholic sodium hydroxide solution at room temperature until no more lignin could be removed by this treatment. The method followed was that described in a previous communication (5). The crude lignin obtained after drying amounted to 63 gm. (6.8 per cent calculated on the weight of the moisture-free and alcohol-benzene-extracted straw). This was treated with 1 liter of an acetone-alcohol solution (2 volumes of acetone to 1 volume of 95 per cent ethanol), the lignin solution was filtered, and the alcohol and acetone were removed by distillation under reduced pressure. The lignin was washed with water, air-dried, and finally dried *in vacuo* at 56° over phosphorus pentoxide. Yield 49 gm. (5.3 per cent calculated on the weight of the moisture-free and alcohol-benzene-extracted straw). The lignin obtained was a light tan amorphous powder. Carbon, hydrogen, and methoxyl determinations made on this material gave the following results: found, C 62.13, 62.20, H 5.93, 5.90,  $OCH_3$  16.42, 16.69. The alkoxy groups present in this lignin fraction were definitely shown to be methoxyls. The method used has previously been described (3).  $C:OCH_3 = 9.70:1$ .

carbon present in the form of methoxyl indicates the number of carbon atoms present in each unit containing one methoxyl group. The above data would indicate that for every unit of 10 carbon atoms there is one methoxyl group.

The foregoing data agree with the formula  $C_{42}H_{48}O_{16}$ , 808, C 62.35, H 5.98. Assuming the presence of four methoxyl groups in a compound of such molecular weight, the calculated percentage is 15.3. The percentage methoxyl actually found in this lignin fraction is somewhat high as compared with that calculated.

*Acetylation*—The lignin was acetylated by the method described in a previous communication (1). The product was dried over solid potassium hydroxide and finally *in vacuo* at 56° over phosphorus pentoxide. The percentage of acetyl was determined by the method described by one of us (8).

*Analysis*—

$C_{42}H_{48}O_{16}$ <div style="display: inline-block; vertical-align: middle; margin-left: 10px;"> <math>\begin{array}{l} \diagup (OCH_3)_4 \\ \diagdown (CO \cdot CH_3)_5 \end{array}</math> </div>	Calculated.	$CH_3CO$	21.1
	Found.	"	21.3, 21.5

*Distillation with 12 Per Cent Hydrochloric Acid*—2 gm. of lignin were distilled with 12 per cent hydrochloric acid according to the procedure recommended by the Association of Official Agricultural Chemists for the determination of pentosans (9). The distillate gave a negative test for furfural with aniline acetate paper. The distillate was neutralized with sodium bicarbonate made slightly acid with acetic acid, then cohobated, and the first 50 cc. of distillate were retained. Formaldehyde was identified in this distillate by the dimethylcyclohexanedione method of Weinberger (10).

*Methylation with Diazomethane*—The methylation with diazomethane was carried out according to the procedure described in a previous communication (6).

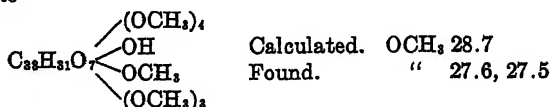
*Analysis*—

$C_{42}H_{48}O_{17}$ <div style="display: inline-block; vertical-align: middle; margin-left: 10px;"> <math>\begin{array}{l} \diagup (OCH_3)_4 \\ \diagdown (OH)_2 \end{array}</math> </div>	Calculated.	$OCH_3$	25.5
	Found.	"	25.0, 25.0

*Methylation With Dimethyl Sulfate*—5 gm. of lignin were dissolved in a solution consisting of 10 gm. of sodium hydroxide in 100 cc. of water and 24 cc. of dimethyl sulfate were added drop by

drop while the reaction mixture was stirred mechanically. After all the dimethyl sulfate had been added, the reaction mixture was heated for half an hour on the steam bath. The methylated product was filtered off and the methylation procedure described above repeated three more times. The product was finally washed with water until free of sulfates, then air-dried, and finally dried *in vacuo* at 56° over phosphorus pentoxide.

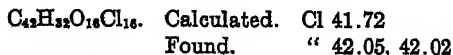
*Analysis—*



*Alkali Fusion*—5 gm. of the first lignin fraction were added to 50 gm. of potassium hydroxide, 25 cc. of water, and 10 gm. of zinc dust contained in a nickel crucible and the fusion of this mixture carried out according to the procedure described in a previous communication (6). The cold fusion product was dissolved in water, the solution was acidified with sulfuric acid and filtered, and the filtrate was extracted with six successive portions of ether. After removal of the ether by distillation, the residual material was dissolved in a 5 per cent sodium carbonate solution, and the alkaline solution was acidified with acetic acid and extracted with ether. The ether was distilled off and the residue crystallized from hot water. The crystals obtained were identified by their melting points and mixed melting points as protocatechuic acid. Yield 0.225 gm. = 4.5 per cent of the weight of the lignin.

*Chlorination*—2 gm. of lignin were suspended in dry carbon tetrachloride and a stream of dry chlorine gas was passed in for about 20 hours. The reaction product was filtered off and dried *in vacuo* at 56°, first over solid potassium hydroxide and then over phosphorus pentoxide.

*Analysis—*



*Isolation of Second Lignin Fraction*—The straw (791 gm.), which had been exhaustively extracted with alcoholic sodium hydroxide solution as described above, was next treated with 4 per cent aqueous sodium hydroxide solution and the second lignin fraction

isolated and purified by the procedure described in a previous communication (6). An amorphous substance was obtained. Yield 10 gm.

*Analysis*—Found. C 61.71, 61.85, H 5.37, 5.50, OCH<sub>3</sub> 17.23, 17.43

This fraction appeared to be similar in composition to the second lignin fraction isolated from barley straw (5). The analytical data obtained in connection with that fraction agreed with that required for the formula C<sub>40</sub>H<sub>42</sub>O<sub>16</sub>.

*Acetylation*—The acetyl derivative was prepared by the method previously described (1). The percentage acetyl was determined by the same method as was used for the analysis of the acetyl derivative of the first lignin fraction (8).

*Analysis*—

C <sub>40</sub> H <sub>38</sub> O <sub>18</sub> · (CH <sub>3</sub> · CO) <sub>4</sub> .	Calculated.	CH <sub>3</sub> CO	18.2
	Found.	"	19.2, 19.5

*Isolation of Residual Lignin*—A portion (50 gm.) of the straw remaining after separation of the second lignin fraction was boiled for 3 hours under a reflux condenser with 1.5 liters of water. The reaction product was filtered and the cellulosic residue was again boiled for 3 hours under a reflux condenser with 1.5 liters of 1 per cent hydrochloric acid solution. The extracted cellulosic material was dried at 105°. Yield 38 gm. This material was finely ground (80 mesh) and added portionwise to 1000 cc. of cold fuming hydrochloric acid, and the lignin isolated according to, in general, the procedure recommended for the quantitative estimation of this substance in plant materials (11). The lignin obtained was brown in color. Yield 1.26 gm. = 2.52 per cent of the weight of the moisture-free residual straw.

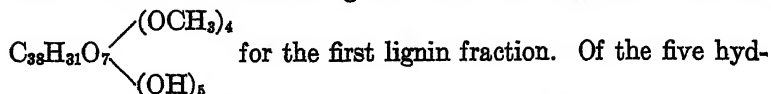
*Analysis*—(Ash-free basis).

Found. C 63.82, 63.90, H 5.31, 5.40, OCH<sub>3</sub> 16.52, 16.40

#### SUMMARY

Three lignin fractions were isolated from wheat straw by subjecting the straw to exhaustive extraction first with a 2 per cent alcoholic sodium hydroxide solution at room temperature, then by refluxing it with a 4 per cent aqueous sodium hydroxide solution, and finally by the treatment of the residue with fuming hydro-

chloric acid. The data agreed with the dissected formula



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## STUDIES ON INFLAMMATION\*

### III. PROTEINASE AND PEPTIDASE ACTIVITY OF POLYMORPHO- NUCLEAR LEUCOCYTES, MONOCYTES, AND EPITHELIOID CELLS OF PLEURAL INFLAMMATORY EXUDATES

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Since the introduction by Willstätter and Bamann (3) of the term cathepsin to designate one of the intracellular proteinases (1929), the literature in this field has grown rapidly. Although reviews have recently appeared by Pozzi (4) and by Maschmann (5), it is necessary to refer to the original papers, especially those of Willstätter, Bamann, and Rhodewald (6), Husfeldt (7), Oelkers and Fischgold (8), Kleinmann and Scharr (9), and Maschmann and Helmert (10) in order to appreciate the reasons for the lack of agreement among the various workers. This is due largely to the multiplicity of animal species selected for study (horse, pig, dog, rabbit, and man), of sources of enzyme material (mixed cells of normal blood, leucocytes of inflammatory exudates, cells from leukemia patients or lymph nodes of animals), of techniques for the preparation of organs and cells preliminary to extraction of enzymes (minced fresh tissue, tissue frozen, dehydrated with acetone and powdered), of solvents employed for extraction of enzymes (glycerol, water, or electrolyte solutions), of substrates (casein, edestin, egg albumin, gelatin, or the tissue proteins associated with the enzyme material itself), of methods for estimation of proteolysis (Van Slyke amino nitrogen determination, formol or alcohol titration, nephelometric and gravimetric methods, etc.), and of types of activators employed (cysteine, enterokinase,  $H_2S$ ,  $HCN$ , etc.).

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For previous studies in this series, see (1, 2).



Recently, however, Anson and Mirsky (11) have done much to clarify the situation by pointing out that casein, edestin, and gelatin are not desirable for use in proteolytic investigations, since they are not reproducible substrates. Furthermore, neither the formol nor the alcohol titration (12) determines exclusively proteinase activity, since both measure at the same time proteolysis by peptidases. In other words, these methods estimate increases in carboxyl (or amino groups) during the hydrolysis not only of proteins, but also of their degradation products. A further improvement was made by Anson (13) in introducing denatured hemoglobin for the estimation of true catheptic proteinase activity. This substrate is rapidly and easily hydrolyzed, requiring only a slight degree of digestion to become non-precipitable by means of trichloroacetic acid. With a short period of digestion there is the additional advantage of avoiding extensive cellular autolysis.

With these advances the opportunity was afforded us to extend our investigations on the proteolytic enzymes of monocytic and polymorphonuclear leucocytes produced in the pleural cavity of rabbits in response to irritants. The present communication deals with the results of these studies, as well as with estimations of dipeptidase and carboxypeptidase activities. In addition, there are included data on a third type of cell, the epithelioid cell, produced by the injection of a phosphatide fraction of the human tubercle bacillus (*Mycobacterium tuberculosis*, H-37).

#### *Methods and Materials*

*Preparation of Exudate*—Polymorphonuclear and monocytic exudates were produced in the pleural cavity of rabbits by methods previously described (2). Animals injected with aleuronat and starch or light mineral oil yielded exudates which, in supravital stained preparations, showed 90 per cent polymorphonuclear leucocytes or 85 per cent monocytes, respectively.

For the preparation of epithelioid cells, Phosphatide A3, derived from a human strain of *Mycobacterium tuberculosis* (H-37)<sup>1</sup> was injected intrapleurally on 3 successive days into each of six rabbits, a dosage of 2 cc. of a 1 per cent saline suspension for each

<sup>1</sup> Mulford Biological Laboratories (Sharpe and Dohme), Glenolden, Pennsylvania, kindly supplied this material.

pleural cavity (14, 15) being employed. The animals were exsanguinated 8 days after the last injection. Supravital stains made of the phosphatide-induced plaques revealed a mass of first, second, and third stage epithelioid cells, but no giant cells. There was also an occasional polymorphonuclear leucocyte and lymphocyte or plasma cell and a few monocytes of the rosette type. These findings are in agreement with the results obtained by Smithburn and Sabin (15).

Polymorphonuclear and monocytic exudates were withdrawn under sterile precautions, immediately centrifuged, and washed three times with Ringer's solution to remove traces of supernatant fluid. Clotting was prevented by the addition of 1 mg. of heparin (Connaught Laboratories, Toronto) per 15 ml. of fluid. The polymorphonuclear material was preserved for future use by dehydrating in the Flosdorf-Mudd lyophile apparatus (16). Monocytic cells were examined shortly after removal from the body of the animal, since the presence of mineral oil interfered with the drying process.

*Preparation of Enzyme Material*—Enzyme solutions were made from polymorphonuclear leucocytes and monocytes, either fresh or lyophile cells being employed, as indicated in Table I, by methods previously described (2). For the estimation of peptidase activity a cellular suspension of lyophile material was used in concentrations of 1 to 3 per cent. The dehydrated cellular residue was pulverized and suspended in water by grinding in a mortar.

*Estimation of Proteolysis*—For the estimation of proteinase activity, denatured hemoglobin (2 per cent) was used as a substrate according to the technique of Anson (13). It was prepared in a series ranging from pH 1.0 to 10.0 by adding varying amounts of 1 N hydrochloric acid or sodium hydroxide to the stock solutions. The stock solution for cathepsin (13) was used from pH 1.5 to 4.5, while that for trypsin (11) was employed from pH 5.0 to 10.0. A hemoglobin solution at pH 1.0 was obtained by diluting a 10 per cent solution with hydrochloric acid and water. All pH measurements were checked with the glass electrode of DeEds (17).

After a series of preliminary determinations, a half an hour period of incubation at 37° was selected as a standard time in-

terval, since the amount of tyrosine liberated and the rate of hydrolysis were sufficiently large under these conditions to give reproducible and satisfactory results.

The intensity of color formed after the addition of phenol reagent to the trichloroacetic acid filtrate was measured with the Evelyn photoelectric colorimeter (18) which had been calibrated with known amounts of tyrosine. The ordinates in Figs. 1 to 3 represent the amount of color-producing material, expressed as micrograms of tyrosin, liberated from 5 cc. of denatured hemoglobin solution and rendered non-precipitable with trichloroacetic acid as a result of the action of 1 cc. of enzyme extract.

For the estimation of the hydrolysis of protamine (salmine)<sup>2</sup> or of *dl*-alanylglycine and chloroacetyl-*l*-tyrosine (Hoffmann-La Roche, Inc.) the formol titration method of Northrop (19) was employed. Titrations were carried out with 0.01 N sodium hydroxide after an incubation period of 4 hours at 37° in a water bath. The standard digestion mixture, which was placed in test-tubes 22 × 175 mm., consisted of the following: (a) substrate solution (2 cc.), a 2 per cent solution of protamine or a 1 per cent solution of the peptides; (b) buffer solution (2 cc.), phosphate buffers (Clark and Lubs) at pH 5.5 or 8.0; (c) activator (1 cc.), cysteine hydrochloride (0.8 per cent) adjusted to pH 5.5 or 8.0 with sodium hydroxide; in control experiments 1 cc. of water was substituted; (d) enzyme solution, 1 cc.

One experiment with casein (prepared according to Hammarsten) was performed with the aid of the alcohol titration of Willstätter and Waldschmidt-Leitz (12). Incubation was for 18 hours at 37° (Table II).

In all experiments, the total amount of hydrolysis was determined both before (at zero time) and after heating the enzyme mixtures in a water bath at 37° and the difference was recorded. Two additional control experiments were performed at the same time. The amount of hydrolysis due to autolysis of enzyme material and to non-enzymatic splitting of substrate was measured in suitably prepared blanks. These values were subtracted from the total hydrolysis. Thus, all figures for enzyme activity were corrected for both autolysis and non-enzymatic hydrolysis of

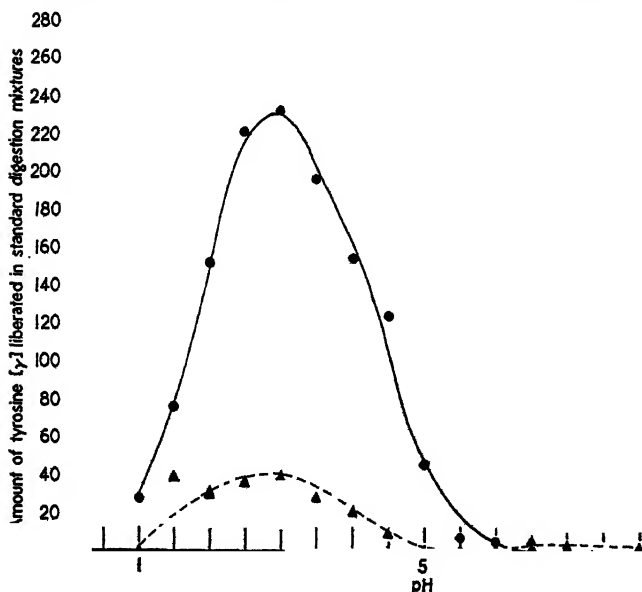
<sup>2</sup> Eli Lilly and Company, Indianapolis, kindly supplied this material.

substrate. These corrections were small in the relatively short digestion periods employed.

Merthiolate in 1:50,000 dilution was employed to inhibit bacterial action.

### Results

Fig. 1 illustrates proteolysis of denatured hemoglobin by means of lyo and desmo extracts of polymorphonuclear leucocytes, as measured by the Anson technique (13). Polymorphonuclear



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FIG. 1. Proteolysis of denatured hemoglobin by extracts of polymorphonuclear leucocytes with varying pH. ● and ▲ represent lyo and desmo preparations, respectively.

leucocytes contain a proteinase which digests hemoglobin from about pH 1 to 5, with an optimum at pH 3.0, the activity dropping off very rapidly on the alkaline side of pH 5. There seems to be no qualitative difference between the enzyme extractable in water (lyo) and the residue which remains in the stroma of the leucocytes (desmo fraction). An inspection of Figs. 2 and 3 shows that extracts of monocytes and epithelioid cells give essentially the same type of curve, except for minor variations.

Anson (13) has called attention to the fact that purified liver cathepsin decreases rapidly in activity on the acid side of pH 2.5. An inspection of Figs. 1 to 3 shows that there is appreciable hydrolysis of hemoglobin, even at pH 2. Whether or not we are dealing with a mixture of two enzymes, namely cathepsin and "pepsin," has not been determined, since there is at present no available method for separating these two enzymes.<sup>3</sup>

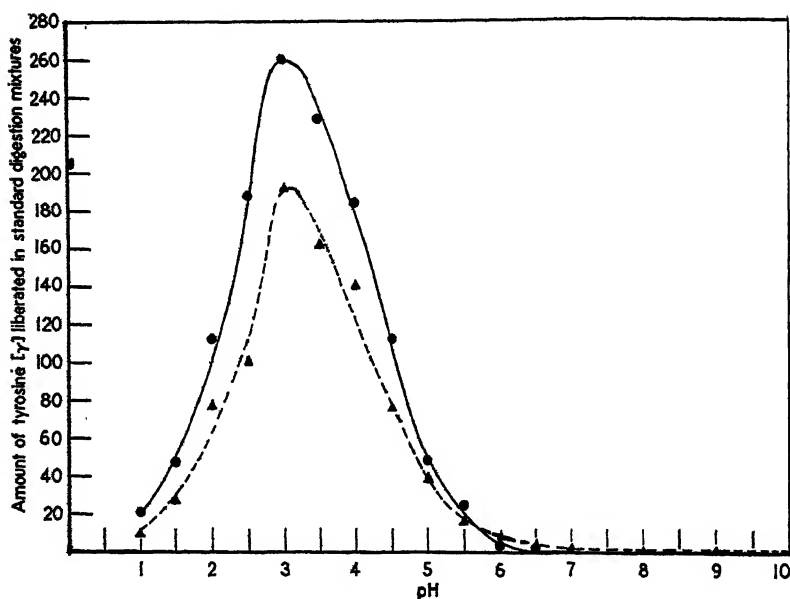


Fig. 2. Proteolysis of denatured hemoglobin by extracts of monocytes with varying pH. ● and ▲ represent lyo and desmo preparations, respectively.

In order to shed further light on this question, an attempt was made to find a substance that would inhibit the action of one enzyme and not the other. Preliminary experiments were performed with purified beef liver cathepsin (13) and pig gastric pepsin (20) with various chemicals such as iodoacetic acid (0.01 M), phenylhydrazine (0.01 M), copper sulfate (0.08 M), and a purified trypsin inhibitor obtained from thin egg white (21). The

<sup>3</sup> Personal communications from Dr. M. L. Anson and from Dr. R. Willstätter.

latter was employed in amounts of 8, 32, 90, and 150 mg. per cc. of enzyme solution, but exerted no inhibitory action. No differentiation between peptic and catheptic activity could be obtained with any of these chemicals.

In a further attempt to demonstrate a difference in activity between pepsin and cathepsin, another type of substrate, protamine, was selected. The data from this laboratory confirm the

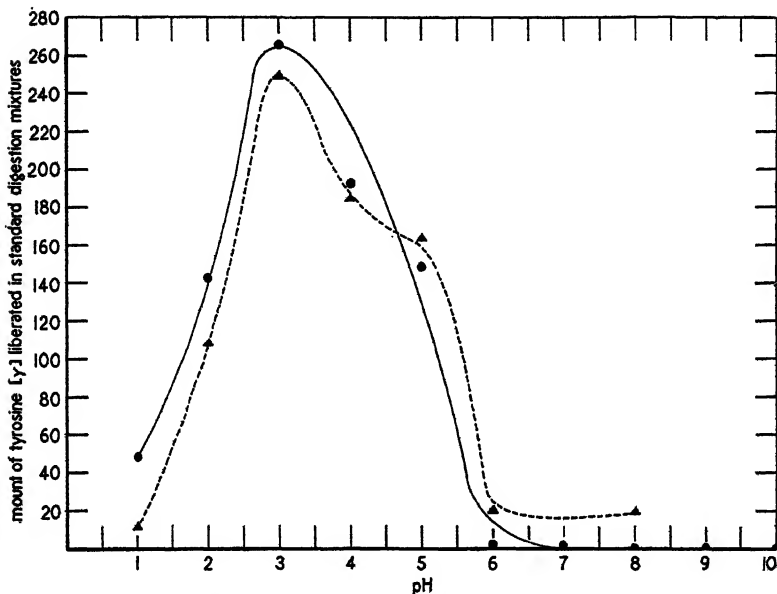


FIG. 3. Proteolysis of denatured hemoglobin by extracts of epithelioid cells with varying pH. ● and ▲ represent lyo and desmo preparations, respectively.

results of Waldschmidt-Leitz (22). Only cathepsin attacked this substrate. When leucocytic extracts, however, were employed as a source of enzyme, no appreciable activity was obtained. This may be due to a much higher concentration of cathepsin in the liver material than in the leucocytes or possibly to the presence of a specific protaminase in the liver preparation.

A comparison of the peptidase activities of suspensions of polymorphonuclear leucocytes, monocytes, and epithelioid cells is shown in Table I. As will be seen, all three types of cells hydrolyze

alanylglycine at pH 8, but only the polymorphonuclears function at pH 5.5. The epithelioid cells resemble the monocytes in the absence of activity at this hydrogen ion concentration. This indicates a qualitative difference between cells of the myelocytic and monocytic series. A carboxypeptidase, capable of hydrolyzing chloroacetyltyrosine at pH 8.0 is absent in all three types of cells.

The addition of cysteine to the digestion mixtures did not increase the enzyme activity of the cellular suspensions, with the single exception of polymorphonuclear leucocytes at pH 5.5.

TABLE I

*Comparison of Peptidase Activity of Suspensions of Polymorphonuclear Leucocytes, Monocytes, and Epithelioid Cells*

The results are expressed in ml. of 0.01 N NaOH.

Type of cell	Chloroacetyl-L-tyrosine, pH 8.0		DL-Alanylglycine			
			pH 5.5		pH 8.0	
	Original	Activated*	Original	Activated*	Original	Activated*
Polymorphonuclear† .....	0.09	0.13	0.57	1.05	5.95	5.79
Monocytes.....	0.07	0.19	0.03‡	-0.03	5.15	5.30
Epithelioid‡.....	-0.25	0.01	0.01	0.05	4.19	3.56

\* 1 cc. of an 0.8 per cent solution of cysteine was added.

† Cells dehydrated by means of the lyophile process prior to measurement of activity.

‡ No activity was observed when the digestion time was extended to 24 hours.

Natural activators are usually present in tissue cells (5, 23, 24) and hence such suspensions rarely require the addition of the type of activators represented by cysteine, reduced glutathione, etc.

Since, in a previous report (2), no evidence was found of a monocytic enzyme active at pH 5.5, it was deemed advisable to repeat this study with gelatin and casein as substrates. With improved methods of extraction, this type of enzyme was demonstrated in both lyo and desmo fractions of the monocytes as well as in the epithelioid cells (Table II). The present data confirm the previous findings that only the polymorphonuclears have





proteolytic activity at pH 8. Monocytes and epithelioid cells are negative in this respect.

#### SUMMARY

With denatured hemoglobin as a substrate (13) an estimation was made of the catheptic activity of three types of phagocytic cells—polymorphonuclear leucocytes, monocytes, and epithelioid cells—which had been produced experimentally in the pleural cavity of rabbits by the injection of aleuronat and starch, light mineral oil, and a phosphatide (A3) derived from *Mycobacterium tuberculosis*, H-37, respectively.

These cells contain a cathepsin with an optimum at pH 3, the curve of activity extending from pH 1.0 to about pH 5.5. They also have in common a dipeptidase which hydrolyzes *dl*-alanylglycine at pH 8 but lack a carboxypeptidase capable of splitting chloroacetyl-*l*-tyrosine at pH 8. The monocytes may, however, be differentiated from the myelocytic type of leucocytes, since only the latter can split *dl*-alanylglycine at pH 5.5 and hydrolyze gelatin and casein at pH 8.

These cellular enzymes exist in an active state and do not require the addition of activators. Indeed, the addition of cysteine does not affect appreciably their activity.

Since the epithelioid cells are, on the basis of embryological researches, derived from monocytes, it is of interest to note that they also have an enzyme pattern which is similar to the latter, and not to the polymorphonuclear leucocytes. This gives chemical support to the morphological studies of Sabin, Doan, and Cunningham (25).

These data suggest application of this method to a study of the immunology of experimental tuberculosis. Work is in progress on the behavior of the cellular proteinases and peptidases in various stages of allergy and immunity, with particular regard to the possible rôle of the specific soluble carbohydrate and phosphatide of the tubercle bacillus and of tuberculin, as activators or inhibitors of enzyme action during caseation and other stages of the infection.

It has incidentally been shown that by means of the Flosdorf-Mudd lyophile apparatus it is possible to preserve tissues for many months, thus permitting a study of their proteinase and peptidase content at one's convenience.

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## ON THE PERCHLORIC-ACETIC ACID METHOD OF AMINO ACID TITRATION\*

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The best method at present available for the exact titrimetric determination of amino acids appears to be the potentiometric formol titration of Dunn and Loshakoff (1) which was shown to be capable of a precision and accuracy of  $\pm 0.1$  per cent. The formol method measures the acid (proton-donor) properties of the amphoteric amino acid molecule,  $\text{RNH}_2\text{COOH} \rightarrow \text{RNH}_2\text{COO}^- + \text{H}^+$ , regardless of whether the acid properties be assigned to an -inium ion ( $-\text{NH}_3^+$ ), according to the zwitter ion theory, or to a carboxyl group according to the classical picture (2). Obviously a method which measures the basic (proton-acceptor) properties of amino acids,  $\text{RNH}_2\text{COOH} + \text{H}^+ \rightarrow \text{RNH}_3^+ \text{COOH}$ , with similar exactness would be a valuable complement to the above formol method. The data to be presented are intended as a contribution to the question whether and to what extent the titration of amino acids with perchloric acid in acetic acid medium shows promise of becoming the desired complementary method. The use of acetous<sup>1</sup> perchloric acid for amino acid titration was originated by Harris in 1929 (3), described by him in detail in 1935 (4), and independently proposed in the same year by Nadeau and Branchen (5).

Aspects of the problem to which special consideration was given in the present work are the following: direct comparison of results with those obtained by Dunn and associates (Amino Acid

\* Aided by a grant from the Leffmann Fund of the Wagner Free Institute, Philadelphia.

<sup>1</sup> The term "acetous" in analogy with "aqueous" seems convenient for indicating the solvent function of acetic acid as contrasted with "acetic" for the acid function.

Manufactures, University of California at Los Angeles) in the formol method, the magnitude of the blank titration value under different conditions, the influence of the presence of water on the end-point and the solubility of different amino acids in the medium of titration. It was found that the difficulties encountered in connection with solubility are readily overcome by the use of a small amount of formic acid. This expedient eliminates the complicating necessity for back titration which was proposed by the preceding authors and which in certain cases is extremely cumbersome owing to slowness of the dissolving action.

#### EXPERIMENTAL

*Reagents and Equipment*—The medium used throughout was glacial acetic acid, Merck, Reagent. This product in a previous investigation (6) had been found to contain about 0.13 per cent water; *i.e.*, a concentration of about 0.07 M. As an auxiliary solvent formic acid (98 to 100 per cent), Eastman Kodak Company, was employed.

By dilution of carefully standardized concentrated perchloric acid (68 to 70 per cent, Technical, G. Frederick Smith Chemical Company) with acetic acid a solution of 1.094 M  $\text{HClO}_4$  and 3.00 M  $\text{H}_2\text{O}$  was prepared. From calculated amounts of this solution and of standardized (6) acetic anhydride the 0.1 N titrating solution was prepared. Its stability is shown by the following data: normality according to standardization with glycine,  $0.10017 \pm 0.00011$  N (four determinations), and 35 days later, after nearly 100 portions had been poured from the flask,  $0.10023 \pm 0.00008$  N (three determinations). An acetous glycine solution of similar concentration was prepared from acetic acid and pure glycine standardized in the manner to be described.

A 0.1 per cent solution of crystal-violet (87 per cent, Coleman and Bell) in acetic acid was used as the indicator, generally in the concentration of 1 drop per 5 cc. of final solution.

A 10 cc. burette (with 0.05 cc. subdivisions) and pipettes of 10 and 12.5 cc. delivery were standardized gravimetrically for acetic acid, with an error of less than 0.1 per cent.

*Blank Titrations*—The color of crystal-violet in acetic acid changes with increasing acidity from blue to green to yellow, passing through intermediate shades (4). In our experience there

is in this range of colors a "pure" blue, a "pure" green, and a "pure" yellow which easily become fixed and reproducible points in the hands of the operator. In actual titration it was found practical to determine both the "green" and the "yellow" point when titrating in the acid direction, while "green" and "blue" were determined in back titrations. Figs. 1 and 2 show the results of blank titrations under different conditions. In the determina-

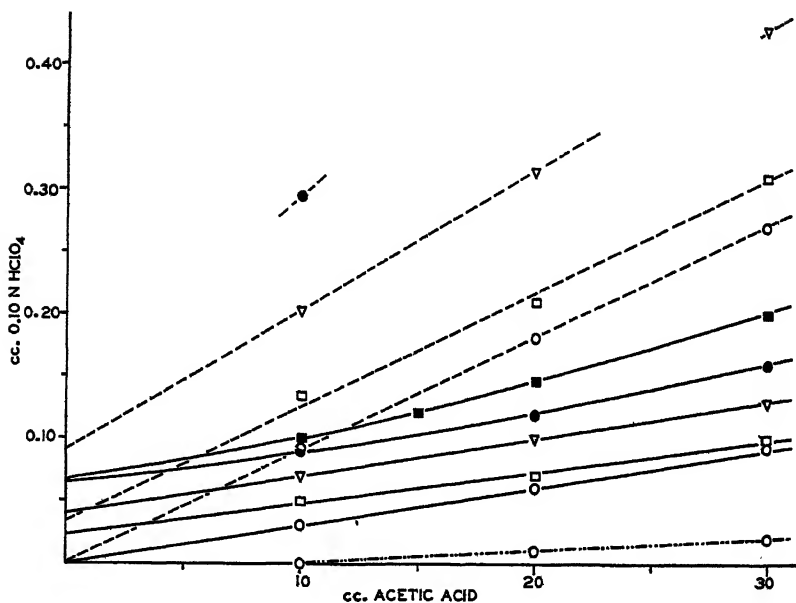


Fig. 1. Blank titrations in acetic acid and the effect of added water.  $\circ$  acetic acid (no water added); acetic acid  $\square$  with 0.2 per cent of added water,  $\triangle$  with 0.4 per cent,  $\bullet$  with 0.6 per cent,  $\blacksquare$  with 1.0 per cent. The solid line represents the green end-point; the dash line, the yellow end-point; the dash and dot line the blue end-point.

tion of thirty points (yellow, green, and blue) covering volumes of acetic acid of 10 to 30 cc., without any added solvent, with addition of 0.2 per cent water, or of 1 or 2 cc. of formic acid, where each point was determined in duplicate, triplicate, or quadruplicate, the total average deviation from the mean value for a single point was  $\pm 0.004$  cc., with a minimum deviation (ten cases) of  $\pm 0.00$  cc. and a maximum (one case) of  $\pm 0.015$  cc. Up to a content of

0.6 per cent of added water the green end-point is of undiminished sharpness, while beginning with 0.4 per cent of added water the yellow end-point becomes increasingly more difficult to define sharply, until in the presence of 1.0 per cent it is impossible to attain, while a reproducible green end-point, although of a somewhat different shade, can still be defined at this water concentration. A study of the data of Fig. 1 shows that the blank titration values are a fairly sensitive index of the presence of water in the medium and that the yellow end-point is more sensitive to its presence than the green one; in other words, that even in the absence of absolute blank values the difference between the green and yellow end-point reveals the presence of water and its quantity.

Accordingly one may expect that in an actual titration the difference between the green and the yellow end-points will be identical with the difference between the two colors obtained in the blank titration of a volume of acetic acid equal to the end-volume of the actual titration, provided (a) that the correct amount of acetic anhydride has been used in the preparation of the perchloric acid solution, so that its residual water content is substantially identical with that of the original acetic acid itself, and (b) that any electrolyte effect due to the presence of the salt (amino acid perchlorate) formed in the titration is negligible as far as the color changes at the end-point are concerned. To test this point 12 and 6 cc. of 0.083 M glycine solution were titrated to the green end-point with 0.10 M perchloric acid solution. Addition of water in amounts equal to 0.4 per cent of the volume (22 and 11 cc.) made necessary the addition of 0.03 and 0.02 cc. of perchloric acid solution in order to reestablish the green end-point. In order to reach the yellow end-point additional amounts of 0.21 and 0.13 cc. were required. The effect of the same amount of water on the green end-point in blank titrations is, according to Fig. 1, 0.04 cc. in both cases, while the blank difference between green and yellow is 0.23 and 0.14 cc., indicating a substantially identical effect of water on the end-points in blank and actual titration.

Fig. 2 shows the effect of small amounts of formic acid on the end-points. Here it was not the effect of constant concentrations of formic acid that was determined but rather the effect of con-

stant amounts (at two levels, 1 and 2 cc.) with varying volumes of acetic acid, since this method of tabulation will furnish the required blank values when in actual titration small volumes of formic acid are used to dissolve the sample prior to the titration. The blank titrations in the presence of 1 or 2 cc. of formic acid are just as satisfactory as those without formic acid as far as the green end-point is concerned, while the yellow end-point tends to become somewhat difficult to define.

*Titration of Amino Acids*—The titrations were carried out in 50 cc. glass-stoppered Erlenmeyer flasks. The amount of amino acid used was generally somewhat less than 1 milliequivalent,

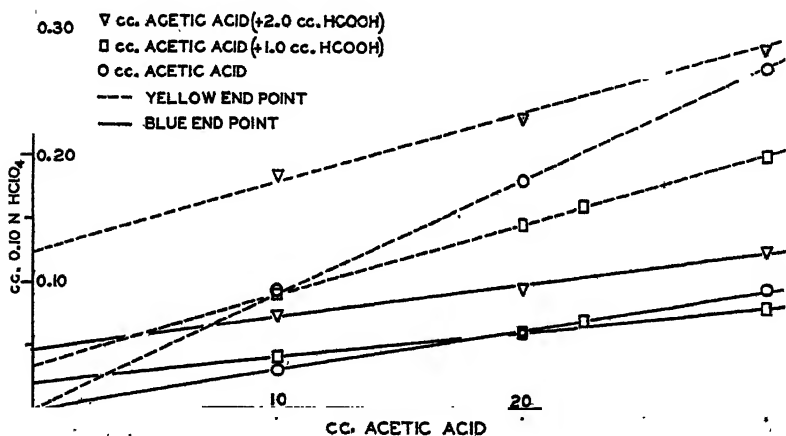


FIG. 2. Effect of formic acid on the blank titration values of acetic acid

corresponding to less than 10 cc. of 0.1 N perchloric acid solution. When the room temperature deviated from 25°, a correction was applied to the titration results in accordance with a thermal expansion coefficient of 0.107 per cent per degree. This figure was derived from the Beilstein data on the density of acetic acid at temperatures between 18–30°. Those amino acids that offered no solubility difficulties were directly titrated in the dry state. In some cases of difficult solubility 10 or 12.5 cc. of perchloric acid solution, corresponding to an excess of 20 or 25 per cent, were added from a calibrated pipette to the dry sample, and when the latter had dissolved, the titration was completed with the stand-



ardized glycine solution. A specimen of glycine, "analytically pure," from Amino Acid Manufactures (Lot 8, stated analyses 99.96 and 100.17 per cent of the calculated value) was used as the primary standard in the present work and its purity was taken as 100.00 per cent. In the standardizations and in all other direct titrations, both the green and the yellow end-points were determined, while in cases of back titration the green and the blue points were used. On subtraction of the corresponding blank values read from the graphs (Figs. 1 and 2), each determination yields two net titration values which theoretically should be identical. Actually in twenty-six direct determinations the mean difference between green and yellow net value was  $+0.01 \pm 0.01$  cc. The corresponding mean value for determinations that contained 1 cc. of formic acid was  $+0.01 \pm 0.02$  cc., while in the presence of 2 cc. of formic acid it was  $+0.03 \pm 0.02$  cc. Since in determinations in the presence of formic acid the yellow end-point appeared to be even more difficult to fix than in the corresponding blank determinations, the green end-point alone was made the basis of calculation in these cases, while in all determinations in which formic acid was absent calculation was based on the mean of the green and yellow net value. When formic acid was present, it also appeared desirable to add extra acetic acid so as to make the final volume about 30 cc., while in direct titrations the volume generally was not larger than 10 cc.

The results of our determinations on different amino acids, together with notes on the procedure followed, are summarized in Table I.

#### DISCUSSION

The present results show that the method investigated is capable of a high degree of precision, but they do not afford a definite basis for the evaluation of accuracy in terms of actual purity, except possibly in a few cases. A sample of glycine, purified by us, gave theoretical figures, showing that it was practically identical with the glycine specimen from Amino Acid Manufactures which served as the standard of reference in our work and which by the formol method of its makers had given results of  $100.05 \pm 0.11$  per cent. In the few other cases in which we used an analyzed product from Amino Acid Manufactures the agreement between

TABLE I  
Summary of Amino Acid Titrations

Amino acid	Perchloric acid consumed	Amino acid	Perchloric acid consumed
	<i>per cent theoretical</i>		<i>per cent theoretical</i>
Glycine (a)	100.00*	<i>dl</i> -Phenylalanine (c)	100.40†
	100.06*		100.43†
<i>L</i> -Alanine (b)	100.27†		100.43†
	100.33†	<i>L</i> -Proline (h)	99.51*
<i>dl</i> -Valine (c)	99.18‡		99.99*
	99.42‡	<i>L</i> -Aspartic acid (i)	98.56¶
<i>d</i> -Valine (d)	99.09*		98.83¶
	99.13*		99.16¶
<i>L</i> -Leucine (e)	96.79§		99.37¶
	96.99§	<i>d</i> -Glutamic acid (j)	99.03**
	97.21§		99.38**
<i>d</i> -Isoleucine (f)	99.99*		99.53**
	100.15*	<i>d</i> -Arginine (b)	93.64‡
<i>L</i> -Serine (g)	99.28		93.82‡
	99.32	<i>L</i> -Cystine (k)	98.90††
	99.37		99.20††

(a) Commercial product, recrystallized four times. (b) Commercial product, recrystallized. (c) From Amino Acid Manufactures, Analytically Pure product. (d) From the Ciba Pharmaceutical Company, purified in this laboratory. (e) From the Ciba Pharmaceutical Company, not purified. (f) Hoffmann-La Roche product, purified in this laboratory. (g) Prepared by resolution of *dl*-serine in this laboratory; dried for 4 hours at 105°. (h) Hoffmann-La Roche product. (i) From Amino Acid Manufactures, Analytically Pure product; showed no loss on drying at 105°. (j) Eastman Kodak product. (k) Merck's Blue Label product.

\* Direct titration.

† Dissolved promptly by slight warming with excess  $\text{HClO}_4$  solution; titrated back with glycine solution.

‡ Dissolved promptly in 1 cc. of formic acid; titrated after dilution with 20 cc. of acetic acid.

§ Dissolved on 30 minutes standing with excess  $\text{HClO}_4$  solution; titrated back with glycine solution.

|| Dissolved promptly by excess  $\text{HClO}_4$  solution; titrated back with glycine solution.

¶ Dissolved on 30 minutes standing with 2 cc. of formic acid; titrated after dilution with 20 cc. of acetic acid.

\*\* Dissolved on 5 minutes standing with 2 cc. of formic acid; titrated after dilution with 20 cc. of acetic acid.

†† Dissolved in 2 cc. of formic acid after several hours; directly titrated, 20 cc. of acetic acid being added near the end-point.

the two methods is less satisfying. Table II summarizes these cases as well as those in which analyses of a like compound (although not the same specimen) are available in the papers of Harris (4) or Nadeau and Branchen (5). The data show agreement within the limits of error between the determination of acid (formol method) and basic (perchloric method) groups in the case of glycine and valine, while in the case of phenylalanine the latter method gave definitely higher, and in the case of aspartic

TABLE II

*Comparison of Amino Acid Analyses by Different Variants of Acetous Perchloric Acid Method and by Potentiometric Formol Method*

Amino acid	Acid or base consumed, per cent of theoretical, with average error			
	Dunn*	Present authors	Nadeau and Branchen (5)	Harris (4)
Glycine.....	100.05 $\pm$ 0.11	100.03 $\pm$ 0.03	100.27 $\pm$ 0.03	99.8 $\pm$ 0.9
d-Alanine....		100.30 $\pm$ 0.03		99.0 $\pm$ 0.6
dl-Valine ....	99.55 $\pm$ 0.13†	99.30 $\pm$ 0.12†		98.8 $\pm$ 0.2
l-Leucine.....		97.00 $\pm$ 0.14		99.6 $\pm$ 0.4
dl-Phenylalanine....	100.05 $\pm$ 0.09†	100.42 $\pm$ 0.02†	99.09 $\pm$ 0.07	99.8 $\pm$ 0.3
l-Proline.....		99.75 $\pm$ 0.24	99.48 $\pm$ 0.08	
l-Aspartic acid.....	100.02 $\pm$ 0.07†	98.98 $\pm$ 0.29†	100.86 $\pm$ 0.14	100.6 $\pm$ 0.3
d-Glutamic acid.....		99.31 $\pm$ 0.19	100.13 $\pm$ 0.18	99.3
d-Arginine....		93.73 $\pm$ 0.09		100.6 $\pm$ 1.9
l-Cystine.....		99.05 $\pm$ 0.15	98.50 $\pm$ 0.13	97.9 $\pm$ 0.8

\* Products obtained from Amino Acid Manufactures, accompanied by individual analyses (potentiometric formol method).

† Identical specimen of the respective amino acid.

acid definitely lower, values than the former. It does seem likely, in view of the doubtless high purity of the materials used, that the deviations are due to methodical errors, rather than to contaminations which might escape detection in the formol method. This view is strengthened by the case of cystine. The material used was, according to optical rotation, at least 99.7 per cent pure, and according to sulfur content 99.8 per cent (S found, 26.57, 26.60, 26.68; calculated, 26.67 per cent). However, cystine

was also the only compound encountered that showed very obvious anomalous behavior at the end-points, the color changes being less sharp than usual, and it was also by far the most difficult one to get into solution. Among the other compounds listed in Table II leucine and arginine were undoubtedly grossly impure, while glutamic acid and alanine may be of doubtful purity.

A study of the available (aqueous) dissociation constants of amino acids (7) shows that in cystine the proton potential of the most acid (or synonymously the least basic) group is distinctly higher than in any other amino acid, its first dissociation constant being of the order of magnitude of  $1 \times 10^{-1}$ , while the corresponding constant is of the order of  $5 \times 10^{-3}$  in the case of the simple neutral amino acids. The acid as well as the basic amino acids, and some special compounds such as phenylalanine or hydroxyproline, show values that approach or exceed  $1 \times 10^{-2}$ . Since on theoretical grounds (8) these relative differences must be expected to be accentuated by the transfer from water to a medium of much lower dielectric constant, such as acetic acid, it does not seem unlikely that the present method gives correct results only in the case of the neutral aliphatic  $\alpha$ -amino acids, while in compounds with more acidic groups deviations arise which it may be possible to overcome by the use of a more acidic indicator.

#### SUMMARY

1. In an investigation of the titration of amino acids by perchloric acid in acetic acid the magnitude of blank values and the effect of water and formic acid on the color changes of crystal-violet have been determined.

2. It has been shown that by the use of small amounts of formic acid it becomes possible to carry out the direct titration of amino acids which dissolve with difficulty in acetic acid.

3. The results of this method have been compared with those obtained by the potentiometric formol titration of Dunn and Loshakoff on identical specimens.

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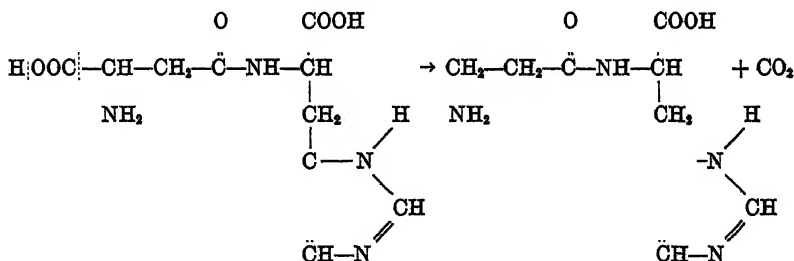
# A PRELIMINARY STUDY OF $\beta$ -L-ASPARTYL-L-HISTIDINE AS A POSSIBLE BIOLOGICAL PRECURSOR OF L-CARNOSINE

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An often mentioned but experimentally untested hypothesis for the metabolic origin of carnosine rests on the suggestion that this  $\beta$ -alanyl peptide of *L*-histidine may arise in the body through the decarboxylation of  $\beta$ -*L*-aspartyl-*L*-histidine.



In order to make possible an experimental approach to this rather attractive hypothesis, we have undertaken the synthesis of this aspartyl peptide and have succeeded in preparing the free crystalline peptide in excellent yield. This was accomplished by condensing the acid chloride of carbobenzoxyaspartic acid  $\alpha$ -benzyl ester with *L*-histidine methyl ester, followed by saponification and reduction. In order to be assured that we actually had the acid chloride of the  $\alpha$ -benzyl ester of carbobenzoxy aspartic acid, the crystalline acid chloride was treated with ammonia. A quantitative yield of the benzyl ester of carbobenzoxyasparagine was obtained, the physical and chemical properties of which agreed with the data of Bergmann, Zervas, and Salzmann (1).

In a recent communication from this laboratory it was shown that carnosine administered subcutaneously was capable of supporting the growth of animals on a histidine-deficient diet (2). It is therefore clear that if the  $\beta$ -l-aspartyl-l-histidine were a precursor of carnosine, it should likewise support growth under these conditions if the rate of conversion be adequate. If a negative result were obtained, one would be justified in eliminating from further serious consideration this hypothesis of the origin of carnosine. Since it was a simple matter to put the question of the growth-promoting powers of  $\beta$ -l-aspartyl-l-histidine to the experimental test, we felt it advisable to undertake such a study before attempting more extensive biological investigations of its proposed rôle as a precursor of carnosine. The results of the feeding trials given in Chart I show clearly that the parenterally introduced  $\beta$ -l-aspartyl-l-histidine behaves like carnosine in supporting growth under the conditions mentioned. These results justify further work on this peptide as a possible biological precursor of carnosine.

The compound was also tested for depressor activity. None was detected under the conditions previously reported (3).

#### EXPERIMENTAL

*Preparation of Carbobenzoxy- $\beta$ -l-Aspartyl-l-Histidine Barium Salt*—16 gm. of histidine methyl ester dihydrochloride were converted to the free methyl ester by the procedure of Fischer and Cone (4). The free ester was dissolved in 150 cc. of absolute chloroform and was cooled to 0°. 11.75 gm. of the freshly prepared acid chloride of carbobenzoxyaspartic acid  $\alpha$ -benzyl ester, prepared according to Bergmann, Zervas, and Salzmann (1), were dissolved in 50 cc. of chloroform and added to the histidine methyl ester solution. An immediate reaction took place. 6.8 gm. of histidine methyl ester monohydrochloride separated from solution. The filtrate was concentrated to a thick oil *in vacuo* and the residue was dissolved in 150 cc. of dioxane. The dioxane solution was again concentrated *in vacuo* to remove the last traces of chloroform. 150 cc. of water and 8.3 cc. of 4 N NaOH were added to the dioxane solution and the mixture was allowed to stand 4 hours at room temperature. It was then neutralized with 8.3 cc. of 4 N HCl. After the solution had been concentrated almost to dryness, the oily residue was washed from the flask with 200 cc. of boiling

water. This solution was made alkaline to phenolphthalein with hot saturated  $\text{Ba}(\text{OH})_2$  solution. The mixture was stirred vigorously until the oily residue was completely dissolved, additional  $\text{Ba}(\text{OH})_2$  being added to keep the mixture alkaline as the oil dissolved. After the mixture had been cooled for 24 hours, the highly insoluble crystalline barium salt which had precipitated was removed by filtration and was washed thoroughly with water, alcohol, and ether. The yield was 9.0 gm. For analysis the compound was dissolved in water containing 2 equivalents of  $\text{HCl}$  and was reprecipitated in crystalline form by the addition of 2 equivalents of  $\text{NaOH}$ .

$\text{C}_{18}\text{H}_{18}\text{N}_4\text{O}_7\text{Ba}$ . Calculated, N 10.38; found, N 10.30

*Preparation of  $\beta$ -L-Aspartyl-L-Histidine*—10 gm. of the barium salt of carbobenzoxy- $\beta$ -L-aspartyl-L-histidine were shaken for 15 minutes with 50 cc. of water and 4 equivalents of  $\text{H}_2\text{SO}_4$ . After the  $\text{BaSO}_4$  had been removed, the solution was reduced with hydrogen in the presence of palladium black. When the evolution of  $\text{CO}_2$  was complete, the catalyst was removed by filtration and the solution was exactly neutralized with  $\text{Ba}(\text{OH})_2$ . The  $\text{BaSO}_4$  was filtered and the solution was concentrated to dryness *in vacuo*. The residue was dissolved in the minimum amount of boiling water. 4 volumes of alcohol were added to the aqueous solution. The solution, upon being cooled, deposited 4.5 gm. of the peptide which crystallized in clusters of needles. The compound melted at  $235$ – $240^\circ$  with decomposition and a 1 per cent aqueous solution had a specific rotation of  $[\alpha]_D^{27} = +38^\circ$ . The air-dried compound contained 2 molecules of water of crystallization. For nitrogen analysis the peptide was dried at  $112^\circ$  *in vacuo*.

$\text{C}_{10}\text{H}_{14}\text{N}_4\text{O}_5 \cdot 2\text{H}_2\text{O}$ . Calculated,  $\text{H}_2\text{O}$  11.74; found,  $\text{H}_2\text{O}$  11.85  
 $\text{C}_{10}\text{H}_{14}\text{N}_4\text{O}_5$ . " N 20.73; " N 20.54

*Growth Experiments*—The histidine-deficient basal diet used in the growth experiments was composed of electrolyzed casein (prepared according to the directions of du Vigneaud, Sifferd, and Irving (2)) 8.6, L-cystine 0.2, L-tryptophane 0.2, dextrin 46.0, sucrose 15.0, lard 19.0, cod liver oil 5.0, salt mixture (Osborne and Mendel (5)) 4.0, and agar 2.0 per cent. Each rat was given twice daily a vitamin supplement of 200 mg. of Yeast Foam Tablet



Powder (Northwestern Yeast Company). The supplements, l-carnosine and  $\beta$ -l-aspartyl-l-histidine, were injected under the

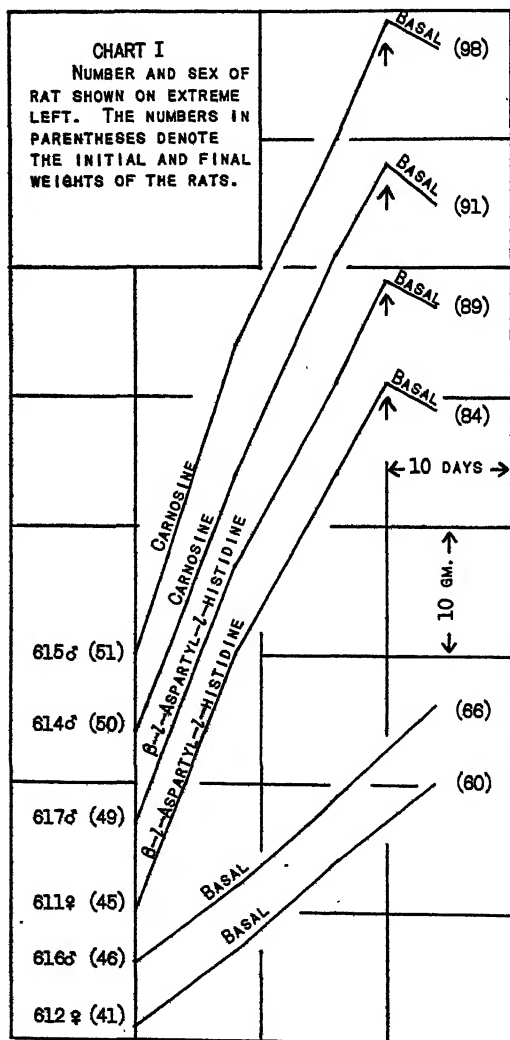


CHART I. Growth curves, showing the comparative rates of growth of rats, in gm., on a histidine-deficient diet supplemented with  $\beta$ -l-aspartyl-l-histidine or with carnosine.

skin of the back of the animal. For the carnosine supplement a solution containing 48.6 gm. of carnosine per cc. was used and 0.5 cc. was injected at 9 a.m., 4 p.m., and 10 p.m., so that each animal received 73 mg. daily. Injections of  $\beta$ -L-aspartyl-L-histidine were made in the same manner and the solution was such that the animals received 99.0 mg. daily. Water was furnished *ad libitum*. The carnosine used was synthesized by the method of Sifferd and du Vigneaud (6).

The growth results are shown in Chart I and the food consumption is given in Table I. Examination of the growth curves will

TABLE I  
*Food Consumption*

Rat No. and sex	Days	Daily supplement to basal diet	Average daily food consumption
		mg.	gm.
612 ♀	1-24		4.2
616 ♂	1-24		3.9
611 ♀	1-20	99 $\beta$ -L-aspartyl-L-histidine	6.7
	21-24		6.7
617 ♂	1-20	99 $\beta$ -L-aspartyl-L-histidine	7.2
	21-24		5.5
614 ♂	1-20	73 carnosine	7.3
	21-24		6.3
615 ♂	1-20	73 carnosine	7.5
	21-24		4.0

indicate that the basal diet employed was not entirely histidine-free but was unquestionably histidine-deficient.

#### SUMMARY

$\beta$ -L-Aspartyl-L-histidine was prepared by condensing the acid chloride of carbobenzoxyaspartic acid  $\alpha$ -benzyl ester with L-histidine methyl ester, followed by saponification and reduction.

This  $\beta$ -aspartyl peptide of histidine has been shown to be capable of supporting the growth of rats on a histidine-deficient diet, similarly to L-carnosine.

The possible rôle of this peptide as a precursor of carnosine in the body has been discussed.

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## N-METHYLCYSTEINE AND DERIVATIVES\*

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In previous studies of the reactivity of cysteine and cystine (1, 2) it has been shown that alkaline decomposition follows an apparently autocatalytic course, and this effect is ascribed to the influence of pyruvic acid produced during the reaction. As it is not observed with derivatives of cysteine containing acyl groups attached to the nitrogen, a highly reactive intermediate compound of the Schiff base type is possibly involved. If this be the case, the autocatalysis should also be suppressed by alkylation of the nitrogen. The synthesis of N-methylcysteine, which has not been reported in the literature, has therefore been undertaken.

Treatment of cystine with dimethyl sulfate (3) or diazomethane (4) leads to the formation of the betaine. Methylation of S-benzylcysteine butyl ester with methyl iodide and of N-benzene-sulfonyl-S-benzylcysteine with methyl sulfate led to products which could not be crystallized. In the procedure which finally yielded the desired compound the di-*p*-toluenesulfonyl derivative of L-cystine was methylated with dimethyl sulfate. The removal of the toluenesulfonyl groups and the reduction of the disulfide linkage were accomplished by treatment with sodium in liquid ammonia, according to the procedure developed by du Vigneaud and coworkers (5, 6). The sulfhydryl compound, precipitated from the reaction mixture as the cuprous mercaptide (7), was converted into the crystalline N-methylcysteine hydrochloride.

N,N'-Dimethylcystine was obtained from the sulfhydryl com-

\* This report is from a dissertation submitted by K. Bloch in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

pound by aeration, but the yield was poor (approximately 60 per cent). Better yields are secured by the action of iodine in acid solution.

The dimethylcystine isolated in the course of the first experiments contained optically inactive material which could only partially be separated by fractional crystallization. It was observed in subsequent experiments that addition of excessive amounts of metallic sodium during the reduction in liquid ammonia causes racemization.

On limiting the proportion of sodium to approximately 7 atoms, the resulting dimethylcystine exhibited a specific rotation of  $+77^\circ$  in  $N$  HCl, and this value could be reproduced in subsequent experiments. This value appears to approximate the maximum rotation of dimethylcystine,<sup>1</sup> although it could not be proved conclusively. In order to ascertain whether any racemization had taken place under the conditions outlined above, dimethyl di-*p*-toluenesulfonylcystine was prepared from dimethylcystine, and its specific rotation compared with that of the same compound obtained by methylation of di-*p*-toluenesulfonylcystine. The rotation of the first compound proved to be 17 per cent lower than that of the latter. This anomalous result may be ascribed to the fact that the toluenesulfonylation of dimethylcystine proceeds very slowly, a 60 per cent yield being obtained only after treatment of dimethylcystine with *p*-toluenesulfonyl chloride in  $N$  NaOH for 20 hours. Under these conditions, 12 per cent racemization occurs with dimethylcystine and 10 per cent racemization with dimethyl di-*p*-toluenesulfonylcystine.

The optical rotation of dimethylcystine, measured at various pH levels and plotted, yielded a curve (Fig. 1) showing the same characteristics as those of *l*-cystine and other naturally occurring amino acids (8).

In the preparation of derivatives of dimethylcystine and methylcystine standard methods were used. Several of the products, notably the acetyl and benzoyl derivatives of dimethylcystine, failed to crystallize. They were studied, however, in the sulfur

<sup>1</sup> According to a private communication from Dr. du Vigneaud the dimethylcystine obtained in his laboratory by a different procedure showed a specific rotation of  $+75^\circ$  (1 per cent in  $N$  HCl at  $22^\circ$ ) and  $+59.5^\circ$  (1 per cent in 0.5  $N$  HCl at  $25.5^\circ$ ).

lability experiments, since the analytical figures indicated sufficient purity.

An attempt to prepare the phenylhydantoic acid from dimethylcystine was unsuccessful. The product formed on treating dimethylcystine with phenyl isocyanate is unstable and undergoes spontaneous ring closure to form the phenylhydantoin. A similar behavior has been observed in the case of N-methyltyrosine, the phenylhydantoic acid of which is likewise unstable.<sup>2</sup>

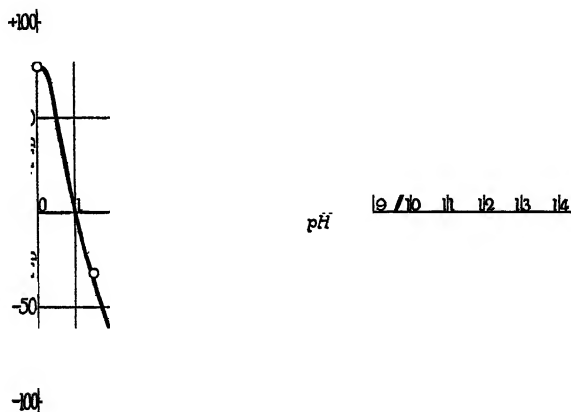


FIG. 1.  $[\alpha]_D$ -pH curve of N,N'-dimethylcystine, 0.5 per cent solution at 25°.

Like cysteine (9), N-methylcystine condenses with formaldehyde in aqueous solution to form the thiazolidine derivative, N-methylthiazolidine-4-carboxylic acid. The tendency to cyclize is, as with phenylhydantoins, markedly greater in the case of the N-methylamino acid. The formation of the thiazolidine derivative can be followed by the increase of the optical rotation; on addition of formaldehyde to the aqueous solution of methylcystine hydrochloride, ring closure occurs to the extent of 90 per cent after 10 minutes at pH 1.5 to 1.6 in the cold, whereas

<sup>2</sup> M. Bovarnick, private communication.

only 20 per cent of thiazolidinecarboxylic acid is formed from cysteine in the same time. The higher stability of the thiazolidine ring in the methyl homologue is indicated furthermore by the fact that the nitroprusside test is negative in *N* alkali, whereas a slight coloration is obtained on applying this test to thiazolidinecarboxylic acid. The titration curve (Fig. 2) displays the ampholytic properties of N-methylthiazolidinecarboxylic acid. It is a strong acid and has the same first dissociation constant ( $pK_1 = 1.5$ ) as thiazolidinecarboxylic acid. On the other hand, it is

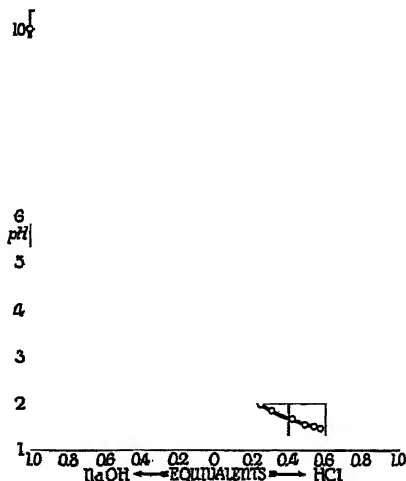


FIG. 2. Titration of N-methylthiazolidine-4-carboxylic acid, 0.05 *M* at 24°;  $pK_1$  1.51,  $pK_2$  6.60.

slightly stronger as a base, the second dissociation constant ( $pK_2 = 6.6$ ) being higher than that of thiazolidinecarboxylic acid (6.2).<sup>3</sup>

The determinations of the lability of sulfur were carried out at 25° under the conditions adopted in previous studies (1, 2). The introduction of a methyl group slightly depresses the initial rate of reaction with alkaline plumbite (Figs. 3 and 4). In the

<sup>3</sup> The pH measurements were made with a glass electrode. The values for the dissociation constants each represent the average of the values calculated from five points of the curve.

cases of cystine and cysteine this rate is maintained at the original level in the presence of hydrazinobenzoic acid (1) or methoxyl-

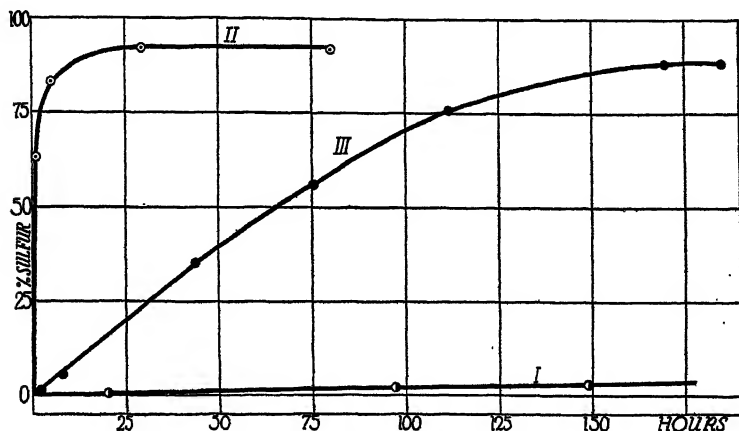


FIG. 3. Labile sulfur from N-methylcysteine derivatives at 25°. Curve I N-methylcysteine, Curve II N-methylcysteine phenylhydantoin, Curve III N-methylacetylcysteine.

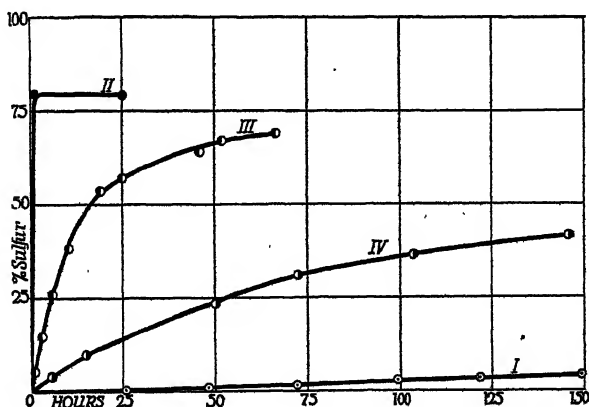


FIG. 4. Labile sulfur from N,N'-dimethylcysteine and derivatives at 25°. Curve I dimethylcysteine, Curve II dimethylcysteine diphenylhydantoin, Curve III dimethyl diacetylcysteine, Curve IV dimethyl dibenzoylcysteine.

amine (2), both of which inhibit the accelerating action of the pyruvate formed during the decomposition. On the other hand,



the N-methylamino acids show no induction period in the elimination of the sulfur, and the addition of salicylaldehyde fails to accelerate the decomposition. The effect observed with cystine and cysteine may therefore reasonably be ascribed to the formation of highly alkali-labile compounds containing carbon doubly linked to nitrogen.

Like cysteine, N-methylcysteine and its derivatives lose their sulfur at a slightly lower rate than the corresponding disulfides. The decomposition rates of the acyl derivatives of dimethylcystine and methylcysteine are of the same order as those of the corresponding unmethylated compounds. The phenylhydantoin of dimethylcystine, like that of cystine, decomposed at a rate too high to measure (Fig. 4).

#### EXPERIMENTAL

*S-Benzylcysteine Butyl Ester*—15 gm. of S-benzylcysteine (10), m.p. 216°, were suspended in 750 cc. of butyl alcohol. Dry HCl was passed through the mixture until the benzylcysteine had dissolved. The solution was refluxed for 2 hours and distilled with frequent addition of butyl alcohol. After no more water distilled over, the solution was concentrated *in vacuo* and yielded long needles of the butyl ester hydrochloride, m.p. 131°.

*Analysis*— $C_{14}H_{22}O_2NSCl$ . Calculated. N 4.6, S 10.6  
Found. " 4.5, " 10.7

*Methylation of S-Benzylcysteine Butyl Ester*—5 gm. of the butyl ester hydrochloride were dissolved in water, and after addition of potassium carbonate, the free ester was extracted with ether. The resulting oil (4.1 gm.) was treated with 20 cc. of benzene and 2.1 gm. of methyl iodide (1.1 moles) in a sealed tube for several days at room temperature. A crystalline precipitate (1.5 gm.) was filtered off; it consisted of the hydriodide of S-benzylcysteine butyl ester, which after removal of hydriodic acid and saponification yielded S-benzylcysteine. The benzene filtrate from the first precipitate was concentrated *in vacuo* and the oily residue saponified by boiling with 5 per cent HCl under a reflux for 4 hours. The aqueous solution on neutralization with ammonia yielded a crystalline precipitate (1.1 gm.), m.p. 198°, which gave analytical figures corresponding to a mixture of unmethylated

and methylated compounds. A separation by fractional crystallization could not be accomplished.

<i>Analysis</i> — $C_{10}H_{13}O_2NS$ .	Calculated.	C 56.8, H 6.2, N 6.6
$C_{11}H_{15}O_2NS$ .	"	" 58.7, " 6.7, " 6.2
	Found.	" 57.8, " 6.7, " 6.4

*S-Benzyl-N-Benzenesulfonylcysteine*—To a solution of 20 gm. of *S*-benzylcysteine in 450 cc. of water and 40 cc. of 8 *N* NaOH, 30 gm. of benzenesulfonyl chloride were added with stirring. After 3 hours, the solution was filtered and acidified strongly with 50 cc. of concentrated HCl. The precipitate (33 gm., or 75 per cent of the theoretical amount) was recrystallized from 60 per cent alcohol; fine needles, m.p. 137°.

<i>Analysis</i> — $C_{15}H_{17}O_4NS_2$ .	Calculated.	N 4.0, S 18.3
	Found.	" 4.0, " 18.3

*Methylation of S-Benzyl-N-Benzenesulfonylcysteine*—A solution of 18.7 gm. of *S*-benzyl-*N*-benzenesulfonylcysteine in 100 cc. of 2 *N* NaOH was stirred with 6.5 gm. of dimethyl sulfate for 1 hour. The clear solution was acidified with hydrochloric acid and the oily precipitate taken up in ether. After the ether was distilled off, the product failed to crystallize. It was treated with metallic sodium in liquid ammonia to remove the benzyl and benzenesulfonyl groups, but no crystallizable material could be obtained from the reaction mixture.

*Di-p-Toluenesulfonylcysteine*—A solution of 48 gm. of *L*-cystine in 1000 cc. of water and 90 cc. of *N* NaOH was shaken with a solution of 152 gm. of *p*-toluenesulfonyl chloride in 400 cc. of ether. A mixture of 50 cc. of *N* NaOH and 600 cc. of water was added in three equal portions during 3 hours. The aqueous layer was acidified with acetic acid, decolorized with charcoal, and strongly acidified with hydrochloric acid. The precipitate (56 gm., 57 per cent of the theoretical amount) was recrystallized from acetone-benzene; large rhombic crystals, m.p. 213–215° (with decomposition).<sup>4</sup>

<i>Analysis</i> — $C_{20}H_{24}O_6N_2S_4$ .	Calculated.	N 5.1, S 23.4
	Found.	" 5.1, " 23.5

<sup>4</sup> McChesney and Swann (11) report a melting point of 201–203° for di-*p*-toluenesulfonylcysteine, recrystallized from 60 per cent alcohol.

*Di-p-Toluenesulfonyl-Di-N-Methylcysteine*—20 gm. of di-*p*-toluenesulfonylcysteine were dissolved in 200 cc. of 2 N NaOH and 23 gm. (2.5 moles) of dimethyl sulfate added. The mixture was stirred and the temperature gradually rose to 42°. After 2 hours stirring, the solution was made acid to Congo red with 10 per cent sulfuric acid. After several hours at 0–5° the oily precipitate was washed free from acid and taken up in chloroform. The chloroform solution was dried over sodium sulfate, evaporated to a small volume, and diluted with twice its volume of benzene. As with dibenzoylcysteine (12), a rigid gel formed, but became crystalline after about 10 days in the ice box. The product was recrystallized from acetone-benzene (19 gm., 91 per cent of the theoretical amount); fine, silky needles, m.p. 125–127°. On recrystallization, the gel was always formed, even when inoculated, and several days were needed for complete crystallization.

*Analysis*— $C_{22}H_{28}O_4N_2S_4$ . Calculated. N 4.9, S 22.2  
Found. " 4.8, " 22.0

*Rotation*— $[\alpha]_D^{25} = +57.7^\circ$  (1.2% in N NaOH)

*N-Methylcysteine Hydrochloride*—To 16 gm. of di-*p*-toluenesulfonyl-di-N-methylcysteine, dissolved in 250 cc. of liquid ammonia, metallic sodium was added in small portions, the first appearance of a transient blue color being taken as the end-point. This occurred when 5 gm. (7 to 8 atoms) of the metal had been added. After addition of 12 gm. of dry ammonium chloride, the solution was allowed to evaporate and the residue dissolved in water, with cooling. Sulfuric acid was added until the solution contained 2 per cent. The turbid solution was shaken several times with ether to remove mercaptans and toluene and freed from ether by aspirating nitrogen. A small amount of sulfur was removed, and the filtrate treated with an excess of freshly prepared cuprous oxide. After about 30 minutes the grayish violet precipitate was centrifuged off and washed several times, until free from inorganic salts. It was then, while still wet, suspended in water and decomposed with  $H_2S$ . The copper sulfide was centrifuged off and the supernatant solution freed from  $H_2S$  by aspirating nitrogen. After the solution was acidified strongly with hydrochloric acid, it was concentrated *in vacuo* at 40°. The resulting syrup was placed in a desiccator over potassium hy-

dioxide. On scratching, the whole mass solidified (5.9 gm., 40 per cent of the theoretical amount); prismatic needles, arranged in bundles. The substance has no sharp melting point; it begins to soften at  $100^{\circ}$  and melts at  $128\text{--}130^{\circ}$  with effervescence.

*Analysis*— $\text{C}_4\text{H}_{10}\text{O}_2\text{NSCl}$

Calculated. C 28.0, H 5.9, N 8.2, S 18.7, Cl 20.9

Found. " 27.6, " 5.7, " 8.2, " 18.9, " 20.7,  $\text{NH}_4\text{-N}$  0.1

*Rotation*— $[\alpha]_D^{25} = +9.21^{\circ}$  (1% in water)

For the iodine titration, 3.648 mg. required 2.16 cc. of 0.01 N  $\text{I}_2$ ; theory 2.17 cc.

*N,N'-Dimethylcystine*—A solution of 5 gm. of N-methylcystine hydrochloride in 200 cc. of water was made alkaline to litmus with ammonia. A small crystal of ferrous sulfate was added and air passed through the solution until the nitroprusside reaction was negative. The solution was acidified with acetic acid and an equal volume of alcohol was added. The precipitate (3.1 gm., 62 per cent of the theoretical amount) consisted of very fine, silky needles, easily soluble in hot water. On recrystallization from 50 per cent alcohol it melted at  $217^{\circ}$  with decomposition.

*Analysis*— $\text{C}_8\text{H}_{16}\text{O}_4\text{N}_2\text{S}_2$ . Calculated. C 35.8, H 6.0, N 10.4, S 23.9

Found. " 35.8, " 5.8, " 10.4, " 24.1

*Rotation*— $[\alpha]_D^{25} = -117.8^{\circ}$  (0.5% in water)

+92.3° (0.5% " N NaOH)

+77.0° (0.5% " " HCl)

In the first experiments racemization occurred during the reduction with metallic sodium in liquid ammonia when 10 to 11 atoms of the metal were used. Dimethylcystine (analysis, N 10.4) isolated from these experiments showed  $[\alpha]_D = +44^{\circ}$  (0.5 per cent in N HCl). On repeated recrystallization from water an optically inactive, less water-soluble fraction (analysis, N 10.5) was obtained; long flat prisms, m.p.  $227^{\circ}$  with decomposition. The more soluble fraction was precipitated from the mother liquor with alcohol; fine silky needles, m.p.  $217^{\circ}$  with decomposition;  $[\alpha]_D = +58^{\circ}$  (0.6 per cent in N HCl). This second fraction still contained inactive material which could not be removed by further recrystallization.

*Dimethylcystine Diphenylhydantoin*—A solution of 1.1 gm. of dimethylcystine in 10 cc. of N NaOH and 13 cc. of water was

shaken with 1 gm. of phenyl isocyanate at room temperature until the odor of the reagent had disappeared. Diphenylurea was filtered off and the filtrate acidified with hydrochloric acid. The precipitate was not homogeneous; the melting point rose continuously on recrystallization and the material became insoluble in alkali. For complete conversion into the phenylhydantoin it was therefore boiled with 10 per cent HCl for 30 minutes under a reflux. After cooling, the solid precipitate (1.5 gm., 72 per cent of the theoretical amount) was recrystallized from glacial acetic acid, m.p. 219–220°.

*Analysis*— $C_{22}H_{22}O_4N_4S_2$ . Calculated. N 11.9, S 13.6  
Found. " 11.9, " 13.8

*Rotation*— $[\alpha]_D^{25} = -131.3^\circ$  (0.7% in chloroform)

*N-Methylcysteine Phenylhydantoin*—A solution of 1 gm. of dimethylcystine phenylhydantoin in 50 cc. of glacial acetic acid was heated on the steam bath in the presence of 1 gm. of zinc dust for 30 minutes. The mixture was filtered and the solution evaporated *in vacuo* to dryness. The residue was taken up in hot dioxane and an equal amount of water added. On cooling, fine prismatic needles separated (0.57 gm., 57 per cent of the theoretical amount), m.p. 179–180°, soluble in cold glacial acetic acid, acetone, and chloroform.

*Analysis*— $C_{11}H_{12}O_2N_2S$ . Calculated. N 11.9, S 13.6  
Found. " 12.1, " 13.4

*Rotation*— $[\alpha]_D^{25} = -101.6^\circ$  (0.3% in chloroform)

In concentrated sulfuric acid at 100° this product yields a stable purple color. The phenylhydantoins of cystine and N-methyltyrosine do not give this test.

*Diacetyl Di-N-Methylcysteine*—Acetylation was attempted first by treating a hot (90–100°) aqueous solution of dimethylcystine with an excess of acetic anhydride. The reaction product obtained on evaporation was a yellowish, brittle, non-crystalline mass. The analytical figures indicated incomplete acetylation. By acetylation of the sodium salt the diacetyl derivative was obtained in good yield, though not in crystalline form.

To a suspension of 1 gm. of dimethylcystine in 5 cc. of water, 18 cc. of 2 N NaOH and 1.9 gm. of acetic anhydride were added with stirring in the course of 30 minutes. An amount of sulfuric

acid equivalent to the NaOH used (6 cc. of 6 N  $\text{H}_2\text{SO}_4$ ) was added and the solution evaporated to dryness under reduced pressure. The residual mixture of inorganic salts and acetyl compound was extracted several times with hot 90 per cent acetone, which dissolves the acetylation product. The acetone solution was filtered and evaporated to dryness; a white, brittle mass resulted which could not be obtained in crystalline condition (1.1 gm., 81 per cent of the theoretical amount).

*Analysis*— $\text{C}_{12}\text{H}_{20}\text{O}_6\text{N}_2\text{S}_2$ . Calculated. N 8.0, S 18.2

Found. " 8.0, " 18.0

*Rotation*— $[\alpha]_D^{25} = -232.8^\circ$  (0.4% in water)

*Acetyl-N-Methylcystine*—To a solution of 1 gm. of diacetyl di-N-methylcystine in 30 cc. of 4 per cent sulfuric acid, 1 gm. of zinc dust was added. The mixture was kept for several hours at room temperature, filtered, and freshly prepared cuprous oxide was added. The precipitated cuprous mercaptide was washed until free from salts and decomposed by hydrogen sulfide. The filtrate was evaporated to dryness *in vacuo*; the syrup solidified on cooling. Prismatic needles were obtained on crystallization from 95 per cent acetone (0.67 gm., 67 per cent of the theoretical amount), m.p.  $132^\circ$ .

*Analysis*— $\text{C}_6\text{H}_{11}\text{O}_3\text{NS}$ . Calculated. N 7.9, S 18.1

Found. " 7.9, " 18.3

*Rotation*— $[\alpha]_D^{25} = -44.5^\circ$  (0.5% in water)

*Dibenzoyl Di-N-Methylcystine*—To a suspension of 1.5 gm. of dimethylcystine in 15 cc. of water and 8 gm. of potassium bicarbonate, 4.5 cc. of benzoyl chloride were added with stirring. The mixture was filtered and acidified to Congo red with hydrochloric acid. The resulting precipitate, consisting of benzoic acid and the benzoyl derivative, was extracted several times with hot ligroin to remove the benzoic acid. The residue was taken up in acetone and twice as much benzene added. The oily precipitate, after drying, formed a colorless, brittle, non-crystalline mass (1.88 gm., 81 per cent of the theoretical amount).

*Analysis*— $\text{C}_{20}\text{H}_{24}\text{O}_6\text{N}_2\text{S}_2$ . Calculated. N 5.9, S 13.5

Found. " 5.8, " 13.2

*Rotation*— $[\alpha]_D^{25} = -220.3^\circ$  (0.5% in alcohol)

*N-Methylthiazolidine-4-Carboxylic Acid Hydrochloride*—To 0.8 gm. of N-methylcysteine hydrochloride in 3 cc. of water 1 cc. of 40 per cent formaldehyde solution was added. After 15 minutes the nitroprusside reaction had disappeared. The solution was placed in a vacuum desiccator and dried over potassium hydroxide. Long prisms of the hydrochloride of the thiazolidine derivative appeared (0.59 gm., 86 per cent of the theory). After recrystallization from alcohol-ether the melting point was 194° with effervescence.

*Analysis*— $C_5H_{10}O_2NSCl$

Calculated. C 32.7, H 5.5, N 7.6, S 17.5, Cl 19.3

Found. " 32.6, " 5.5, " 7.7, " 17.7, " 19.4

*Rotation*— $[\alpha]_D^{25} = -119.2^\circ$  (1% in water)

The free methylthiazolidinecarboxylic acid could not be obtained in a pure state. Owing to its great solubility it could not be separated from pyridine hydrochloride, after addition of pyridine to remove the hydrochloric acid. When silver carbonate was used to remove HCl, the product was crystalline but did not show the required analytical figures.

*Analysis*— $C_5H_9O_2NS$ . Calculated. N 9.5, S 21.8

Found. " 8.6, " 19.5

#### SUMMARY

1. N-Methylcysteine and N,N'-dimethylcystine have been prepared from *l*-cystine. Several of their derivatives have been described.

2. The introduction of a methyl group on the nitrogen atom has no pronounced effect upon the initial decomposition rate in alkaline plumbite, compared with that of the unmethylated compounds, but it inhibits the autocatalytic acceleration of decomposition observed with the latter. This acceleration, known to be due to the effect of pyruvate, must therefore be ascribed specifically to the formation of highly alkali-labile compounds of the Schiff type which cannot take place with the N-alkyl amino acids. This conforms with the previous observation that the decomposition rates of acyl derivatives of cystine and cysteine are unaffected by salicylaldehyde.

3. Like cysteine, N-methylcysteine condenses with formaldehyde to form N-methylthiazolidine-4-carboxylic acid. Its ring system is more stable than that of the corresponding unmethylated compound.

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## THE ERGOT ALKALOIDS

### XIV. THE POSITIONS OF THE DOUBLE BOND AND THE CARBOXYL GROUP IN LYSERGIC ACID AND ITS ISOMER. THE STRUCTURE OF THE ALKALOIDS

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In our previous studies (1) it has been shown that lysergic acid is a cleavage product formed on alkaline hydrolysis of all of the alkaloids which are characteristic of ergot. Since this acid is unique and occurs in these alkaloids conjugated with amino acids (or with hydroxyisopropylamine in the case of ergometrine), it was concluded that lysergic acid itself or perhaps an isomer must be responsible for the unique pharmacodynamic properties of these alkaloids. It was then shown (2, 3) that the double bond of lysergic acid functions in some way in the isomerism of the levorotatory biologically active alkaloids and the dextrorotatory inactive alkaloids, since the dihydro alkaloids lost this property. In conformity with this, it was also noted that the hydrogenated levorotatory alkaloids of the ergotoxine series gave  $\alpha$ -dihydrolysergic acid on hydrolysis, whereas the isomeric dextrorotatory alkaloids yielded the isomeric  $\gamma$ -dihydrolysergic acid (2, 3). From the first, we were inclined to regard lysergic acid itself as the component of the active alkaloids, since lysergic methyl ester mutarotated in solution, becoming more strongly dextrorotatory. However, an uncertainty persisted, since lysergic acid on hydrogenation was found to yield not only  $\alpha$ -dihydrolysergic acid, but also (although in smaller amount)  $\gamma$ -dihydrolysergic acid. In our subsequent, unpublished attempts to clarify this point it was soon found that lysergic methyl ester, unlike lysergic acid itself, behaved on hydrogenation like the levorotatory

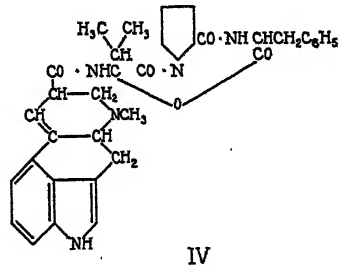
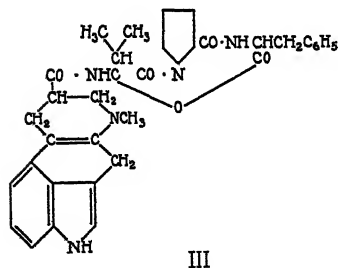
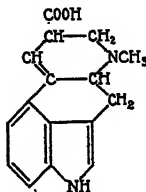
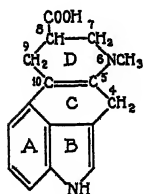
alkaloids of the ergotoxine series. From the reaction mixture only  $\alpha$ -dihydrolysergic acid could be isolated and no  $\gamma$ -dihydrolysergic acid. Thus direct evidence was obtained that the active alkaloids of the ergotoxine-ergometrine series must be derivatives of lysergic acid itself. The simultaneous formation of  $\gamma$ -dihydrolysergic acid with the  $\alpha$  acid from lysergic acid must be explained apparently by the more ready partial isomerization of the latter under the conditions of the hydrogenation.

In the meantime, additional evidence that lysergic acid occurs as such in the active alkaloids has been presented from another angle in the recent reports of Stoll and Hofmann (4). Also the report of the isolation by Smith and Timmis (5) (which anticipated work that was naturally already in progress in our laboratory) of a very strongly dextrorotatory acid, isolysergic acid, by the isomerization of lysergic acid made it appear probable that this isomer is the form which occurs in the dextrorotatory alkaloids of the ergotinine type.

On the basis of degradation studies a tetracyclic structure for lysergic acid has been derived, as given in Formula I (2, 3). Such a structure has been supported by the properties of synthetic substances possessing this ring structure ((6) and unpublished work). In the formula given, the position of the carboxyl group, with certain limitations, remained tentative. Similarly, questions concerning the manner in which the double bond functions in the isomerism of the two series of alkaloids remained to be determined. Comparison of the ultraviolet absorption spectra (7) of lysergic acid and the parent alkaloids with those of the hydrogenated substances indicated quite conclusively that the double bond must be conjugated with the benzene (or pyrrole) ring of the indole nucleus and therefore must be present in the hydroquinoline portion. It was still uncertain whether its position remains the same in the two series of alkaloids and the isomerism is due to different configurations on an asymmetric carbon atom, or whether the double bond occupies different positions in each series of alkaloids—in other words, whether the isomerism is purely stereochemical or structural. In either case it must be concluded that the double bond can undergo change of position quite easily. Certain evidence will be presented in the present report bearing on the question of the exact position of the double

bond in each series of alkaloids as well as that of the carboxyl group in lysergic acid.

It is well known that the proximity of a double bond influences the basicity of a basic group and the acidity of an acid group (8). With this in mind we have made a study of the dissociation constants of a number of derivatives of lysergic acid in the hope that such data would throw light on the remaining uncertainties of lysergic acid.



Since the interpretation of effects is somewhat more difficult when both a basic and an acid group are present, substances were first studied in which the acid group is covered. The three alkaloids ergometrine, ergometrinine, and dihydroergometrine proved to be suitable for this purpose because of their sufficient solubility in water. In each of these substances the carboxyl group is joined to hydroxyisopropylamine in amide linkage and therefore cannot act as an acid. Conversely, basic properties of hydroxyisopropylamine are essentially removed by acylation. The  $\text{CH}_2\text{N}=\text{}$  group is the only basic group present in the molecule, since the indole nitrogen, as is well known, lacks basic properties. The basic apparent dissociation constants obtained from pH

measurements made at the mid-points of the titration curves are given in Table I.

TABLE I  
*Dissociation Constants of Lysergic Acid and Its Derivatives*

	pK' <sub>2</sub> (pH half neu- tralized with NaOH)	Tempera- ture	pK' <sub>1</sub> (pH half neu- tralized with HCl)	Tempera- ture
		°C.		°C.
Lysergic acid	7.96	24	3.19	24
	7.68	24	3.44	24
	7.70*	38	3.20*	38
	7.75	38	3.46	38
Isolysergic acid	8.31	24	3.21	24
	8.61	24	3.44	24
	8.40	38	3.50	38
$\alpha$ -Dihydrolysergic acid	8.45	24	3.57	24
	8.10	38	3.70	38
	8.57	24	3.60	24
$\gamma$ -Dihydrolysergic "	8.64	24		
	pK' (pH half neu- tralized with HCl)			
Ergometrine	6.68	24		
	6.91	24		
	6.60	24		
Ergometrinine	7.26	24		
	7.38	24		
Dihydroergometrinine	7.30	24		
	7.46	24		
$\alpha$ -Dihydrolysergol	8.30	24		
$\beta$ -Dihydrolysergol	8.23	24		
6-Methyl ergoline	8.84	24		
	8.89	24		
	8.87	24		

\* Measured at twice the concentration.

These figures show ergometrine to be a weaker base than ergometrinine. The difference appears to be essentially due to the effect of the position of the double bond in relation to the NCH<sub>3</sub> group. This interpretation is supported, as later discussed, by

the measurements made on lysergic acid, isolysergic acid, and their dihydro derivatives.

Examination of Formula I will show that the only positions possible for the double bond are (4-5), (5-10), (10-9), (9-8), and (8-7). Regardless of where the carboxyl group is placed, positions (9-8) and (8-7) are incompatible with absorption spectra studies (7) which have shown that the double bond must be conjugated with the indole nucleus in some way.

If we turn to positions (4-5) and (5-10) in which the double bonds are equidistant from the  $\text{NCH}_3$  group, to explain the difference between the two isomers, the experimental evidence appears to be against such a view. The fact that ergometrine is the weaker base and that there is little difference between ergometrine and dihydroergometrine<sup>1</sup> makes it necessary to conclude that the double bond in ergometrine must be further removed from the  $\text{NCH}_3$  group than in ergometrine. Therefore position (4-5) or (5-10) appears excluded in the case of this alkaloid. The only position remaining to meet the requirements for this alkaloid is (10-9). Conversely, the double bond of the ergometrine series (*i.e.* lysergic acid) must be located at position (5-10).<sup>2</sup>

Similar conclusions are possible from measurements which have been made with lysergic acid, isolysergic acid, and the dihydro-lysergic acids. In these cases since both acid and basic groups are free, conditions are met similar to those noted with amino acids, which can therefore best be interpreted in terms of the zwitter ion theory. If according to this concept we take as the basic apparent dissociation constant the pH measured when the substance is half neutralized with sodium hydroxide ( $\text{pK}'_2$ ), we obtain the values given in Table I.

Again as in the case of the alkaloids it is found that the basic group of lysergic acid is weaker than that of isolysergic acid. The average difference at 24° is seen to be about 0.64 of a unit in both series of determinations. Also the strengths of the basic

<sup>1</sup> Dihydroergometrine was not available for these studies.

<sup>2</sup> Although this appears to leave open position (4-5) for the double bond of lysergic acid, position (5-10) remains definitely to be preferred for a number of reasons. We hope to obtain conclusive evidence of this by work which is now in progress.

group in the dihydro acids and isolysergic acid show a close approximation. Since we can now accept that ergometrine (the ergotoxine series) is derived from lysergic acid while ergometrinine (the ergotinine series) is derived from isolysergic acid, the values found are consistent with those found for the alkaloids themselves.

In the case of the acid dissociation constants,  $pK'_1$  (the pH measured when the substances were half neutralized with HCl), no significant difference was noted between lysergic and isolysergic acids. On passing to the dihydro acids in each case a slight weakening became apparent.

Certain inferences in regard to the position of the carboxyl group can now be drawn. In order to permit the formation of the quinoline betaine tricarboxylic acid,  $C_{14}H_8O_3N$  (9, 2), and also in order to furnish a necessary asymmetric carbon atom in lysergic acid, the only positions of the carboxyl group which are possible are positions (4), (9), (8), and (7) (Formula I). Of these, position (9) appears eliminated, since migration of the double bond between positions (5-10) and (10-9) should cause ready racemization.

Position (4) appears unlikely on biogenetic grounds because of the inferred relationship to tryptophane. It is still less likely on chemical grounds. Although when heated at low pressure above  $200^\circ$ , lysergic acid loses carbon dioxide and methylamine, dihydrolysergic acid, on the contrary, can be sublimed at  $300^\circ$ . Position (4) for the carboxyl group would make the latter a substituted indoleacetic acid which should lose carbon dioxide readily on pyrolysis. There remain for consideration only positions (7) and (8). Of the two, position (8) appears to be indicated by a comparison of the relative magnitudes of the basic dissociation constants of  $\alpha$ -dihydrolysergic acid and synthetic 6-methyl ergoline (decarboxydihydrolysergic acid) (6). It should be mentioned in passing that the values found for the dihydrolysergols are in agreement with what should be expected from such a hydroxy derivative of 6-methyl ergoline.

From the data given in Table II it can be seen that substitution of a COOH group in an aliphatic amine in a position  $\alpha$  to the  $NH_2$  group reduces the basic dissociation constant by about 1.0 unit, while substitution in the  $\beta$  position diminishes it by about 0.5 unit. The average value of the dihydrolysergic acids (8.55 at  $24^\circ$ ) was

found to be 0.32 unit lower than that of 6-methyl ergoline which is in best agreement with a carboxyl group in the  $\beta$  position or position (8). Similarly the magnitude of the dissociation constant of dihydroergometrine likewise indicates  $\beta$  substitution.

If we consider the dissociation constants of the series ethylamine (10),  $\beta$ -alanine ethyl ester (11), and alanine ethyl ester (11) (Table II), the relative effect of the carbethoxyl group on the basicity of the amino group can be seen. The COOR group is very negative in character, having approximately the same influence as a CON= group, as can be seen from the fact that glycine ethyl ester has the same dissociation constant as glycyglycine ethyl ester (11). From this series it is evident that a COOR group

TABLE II

	pK' (pH half neutralized with HCl)	
Ethylamine.....	10.7	
Alanine ethyl ester.....	7.80	
$\beta$ -Alanine " ".....	9.13	
6-Methyl ergoline.....	8.87	
Dihydroergometrine.....	7.30, 7.46	
	pK' <sub>2</sub> (pH half neutralized with NaOH)	pK' <sub>1</sub> (pH half neutralized with HCl)
Alanine.....	9.72	2.39
$\beta$ -Alanine.....	10.19	3.60
$\alpha$ -Dihydrolysergic acid.....	8.45	3.57, 3.70
$\gamma$ -Dihydrolysergic ".....	8.57, 8.64	3.60

in the  $\alpha$  position diminishes the value of pK' for ethylamine by 2.9 units, while in the  $\beta$  position the decrease is 1.57 units. When the average value of dihydroergometrine, 7.38, is compared with that of 6-methyl ergoline, 8.87, it is found that the introduction of the CONHR group has reduced the pK' by 1.49 units. This is in good agreement with the effect noted for such a substituent in the  $\beta$  position.

A further indication of the  $\beta$  position is to be seen in the magnitude of the acid dissociation constant (pK'<sub>1</sub>) of the dihydrolysergic acids, viz. 3.6 (average), which is in good agreement with that of  $\beta$ -alanine (3.6) (11), while that of alanine and other  $\alpha$ -amino acids is roughly 2.3 (11).



It is therefore provisionally concluded that lysergic acid is represented by Formula I and isolysergic acid by Formula II. The pharmacologically active alkaloids of the ergotoxine group are derivatives of lysergic acid, while the very strongly dextro-rotatory isomeric alkaloids of the ergotinine group are derived from an isomeric lysergic acid. The completed formula of ergotoxine would therefore be most satisfactorily represented by Formula III and that of ergotinine by Formula IV. By replacement of the  $\alpha$ -hydroxyvaline by  $\alpha$ -hydroxyalanine in these formulas, the formulas of ergotamine and ergotaminine are represented respectively. Finally, by replacing the terminal phenylalanine in the latter by *l*-leucine, the formulas of ergosine and ergosinine are obtained.

Further work is naturally in progress to ascertain the validity of these conclusions regarding the structure of lysergic acid, its derivatives, and the parent alkaloids.

#### EXPERIMENTAL

*Preparation of Isolysergic Acid*—The method of preparation of the crude acid was essentially that reported by Smith and Timmis (5). However, since it was found difficult to repeat the purification of the product by their method, the following procedure was found to give sharply and rapidly the desired result. Although in accordance with this the acid can be sharply separated from lysergic acid by crystallization from dilute ammonium hydroxide, it was found impossible to effect similar separation by crystallization from equivalent amounts of dilute acetic acid or by fractional precipitation from the hydrochloride.

1.630 gm. of lysergic acid were boiled in an atmosphere of hydrogen for 6 hours. The solution was treated with a little bone-black to remove the color and the filtrate was cooled in ice. 0.9 gm. of crystals was collected which showed a rotation of  $[\alpha]_D^{25} = +155^\circ$  ( $c = 0.6$  in pyridine). The filtrate was quickly evaporated under reduced pressure to about 40 cc. and the crystalline material which separated was collected after cooling. 0.7 gm. of additional material was collected which had a rotation of  $[\alpha]_D^{25} = +183^\circ$  ( $c = 0.57$ ).

The two fractions were combined and treated with 90 cc. of water to which were added 7 cc. (1 equivalent) of *N* ammonium

hydroxide. The mixture was warmed sufficiently for complete solution and was then quickly cooled in ice. 0.67 gm. of crystalline material was collected after cooling for several hours in ice. The rotation was  $[\alpha] = +282^\circ$  ( $c = 0.5$  in pyridine for the anhydrous substance), which is in good agreement with that reported by Smith and Timmis ( $[\alpha]_D = 281^\circ$ ). A repetition of the above treatment with dilute ammonia did not raise the rotation of the recovered substance.

*Determination of Dissociation Constants*—The materials used in obtaining the data given in Table I were prepared according to directions given by us in previous publications, with the exception of isolysergic acid. All gave correct analyses, rotations, and melting points.

The pH values were obtained with the glass electrode (12) with solutions half neutralized and then made up to volume. In the case of comparisons between isomers, equal weights of substances were treated with the calculated 0.5 equivalent of standard acid or base from a microburette. On check runs, a full equivalent of acid or base was employed and the solution was brought back to the half neutralization point by addition of 0.5 equivalent of standard base or acid. Such a procedure insured that all would be in solution when the concentration was just above saturation. The solutions were then diluted to equal volumes. The solutions were roughly 0.002 N with respect to the substance being measured.

For example, 3.0 mg. of  $\alpha$ -dihydrolysergic acid were treated with 0.108 cc. of 0.1028 N HCl. After solution by addition of a fractional volume of water, 0.055 cc. of 0.1016 N NaOH was added and the volume was made up to 5 cc.

Measurements of pH were made at  $24^\circ$  and also at  $38^\circ$  and in a number of cases checked after dilution.

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## THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

### LIII. STUDIES ON THE PHOSPHATIDE OF THE HUMAN TUBERCLE BACILLUS\*

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In a previous report from this Laboratory it was shown that the phosphatide fraction from the human type of tubercle bacillus, Strain H-37, when hydrolyzed with dilute sulfuric acid, yielded some 66 per cent of fatty acids and about 33 per cent of water-soluble compounds (1). The water-soluble compounds, in addition to inorganic phosphoric acid and glycerophosphoric acid, consisted of inosite (2), mannose (3), and some other hexose, presumably glucose because it gave a typical glucosazone (4). Saponification of the phosphatide with dilute alcoholic potassium hydroxide led to quite different cleavage products (5). The alcoholic solution contained the potassium salts of the fatty acids, while an alcohol-insoluble substance remained which was found to consist of an organic phosphoric acid and a phosphorus-containing polysaccharide. The polysaccharide, which was named mannosinose, gave on hydrolysis with dilute sulfuric acid phosphoric acid, mannose, and inosite. Since this was the first time that a substance had been described which gave inosite and mannose on hydrolysis, we were interested in studying more fully the properties of this unique compound and in determining whether

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it was a glycoside or whether the inosite and mannose were combined as esters of phosphoric acid.

In the work (5) reported several years ago the phosphatide had been saponified by prolonged heating with alcoholic potassium hydroxide and this drastic treatment might have changed the carbohydrate molecule.

In the experiments forming the subject of the present report very mild alkali treatment at room temperature was employed, but the cleavage products were essentially the same as were found in the first experiment. The fatty acids were split off and an organic phosphoric acid together with a phosphorus-containing polysaccharide separated from the solution. The phosphorus was split off by heating a solution of the polysaccharide in dilute ammonium hydroxide in a sealed tube to 170° for 8.5 hours. The dephosphorylated substance gave no reduction with Fehling's solution until after it had been boiled with dilute acid, thus indicating that it was a glycoside. When the glycoside was hydrolyzed by boiling with dilute sulfuric acid, the maximum reduction, as determined by the Shaffer-Hartmann method (6), was attained in 2.5 hours and amounted to 63 per cent, calculated as glucose. The only cleavage products that could be isolated were mannose and inosite and the amounts obtained would indicate that the glycoside, for which we retain the name manninositose, is a tri-glycoside containing 2 molecules of mannose combined with 1 molecule of inosite.

A substance very similar to and probably identical with manninositose was isolated from the polysaccharide of the avian tubercle bacillus by du Mont and Anderson (7). The avian tubercle bacillus glycoside was present, however, in the free state and not combined with phosphoric acid.

In the present study no evidence whatever was obtained of the presence of any hexose except mannose. However, in earlier analyses in which the phosphatide had been hydrolyzed with dilute sulfuric acid three carbohydrates, namely inosite, mannose, and some other hexose which gave a glucosazone after the mannose had been removed as phenylhydrazone, were always found (5). It is evident therefore that direct acid hydrolysis of the phosphatide yields different hexoses than are obtained after the phosphatide has been saponified with dilute potassium hydroxide.

## EXPERIMENTAL

The phosphatide had been prepared from the human type of tubercle bacillus and purified as described in a former paper (8). The substance was a white amorphous powder containing about 3.0 per cent of phosphorus and 0.3 per cent of nitrogen. It was readily dispersed in water, forming colloidal solutions. The concentrated solutions were cloudy but became perfectly clear on sufficient dilution with water. The aqueous solutions gave no reduction when boiled with Fehling's solution but after they were heated for some time with dilute acid reducing sugars were liberated.

*Saponification of the Phosphatide*—The phosphatide, 11.4 gm., was dissolved in 100 cc. of benzene, in which it gave a perfectly clear, faintly yellowish solution, and 1.5 gm. of potassium hydroxide dissolved in 10 cc. of absolute alcohol were added. On standing at room temperature the solution turned cloudy and a gelatinous precipitate separated slowly. After 24 hours the precipitate was filtered off and washed carefully with benzene and with ether and dried *in vacuo*. The filtrate on standing for another 24 hours deposited a small amount of insoluble matter which was filtered off, washed as before, and dried *in vacuo*. The total yield of benzene-insoluble matter was 6.48 gm. or 56.8 per cent of the phosphatide.

*Examination of the Benzene-Insoluble Substance*—The materials which had separated from the benzene solution were dissolved in 100 cc. of water, giving a cloudy solution which was strongly alkaline in reaction. Acidification with acetic acid gave a heavy precipitate which was removed by filtration, washed with water, and dried *in vacuo*. It formed a somewhat sticky mass which weighed 1.6 gm. and was found to consist of fatty acids.

The filtrate and washings were concentrated *in vacuo* to a thick syrup and the syrup was dehydrated by grinding under absolute alcohol in a mortar until a fine white powder was formed. This product, which represents the crude polysaccharide, weighed 4.6 gm., corresponding to 40.3 per cent of the phosphatide.

*Isolation of the Fatty Acids*—The benzene solution was freed of excess potassium hydroxide by means of carbon dioxide and the potassium carbonate was filtered off, washed with benzene, and discarded. The filtrate was concentrated to dryness *in vacuo*

and the residue was dissolved in ether. The ethereal extract was washed, first with dilute hydrochloric acid and then with water, after which it was dried over sodium sulfate, filtered, and evaporated to dryness. The oily residue weighed 5.7 gm. This material was combined with the fatty acid fraction mentioned above, namely 1.6 gm., thus giving a total of 7.3 gm. of fatty acids corresponding to 64 per cent of the phosphatide. Both of these fractions of fatty acids were tested for phosphorus with completely negative results. The fatty acids were reserved for a future study.

*Examination of the Crude Polysaccharide*—A portion of the crude polysaccharide was hydrolyzed with dilute sulfuric acid, after which the solution was extracted with ether. The ethereal extract on evaporation to dryness left the merest trace of a residue, thus indicating that all the fatty acids had been split off by the alkali treatment.

The crude polysaccharide on analysis was found to contain 6.20 per cent of phosphorus, 0.12 per cent of nitrogen, and 29.6 per cent of ash.

*Separation of an Organic Phosphoric Acid from the Crude Polysaccharide*—The remaining portion of the crude polysaccharide, 4.5 gm., was dissolved in 50 cc. of water and a solution of neutral lead acetate was added until no further precipitate occurred. The precipitate was filtered off, washed with water, and decomposed with hydrogen sulfide. The lead sulfide was filtered off; the filtrate was concentrated *in vacuo* to a small volume and neutralized with barium hydroxide. The solution was filtered and the barium salt was precipitated with alcohol. After the substance had been reprecipitated in the same manner, 0.21 gm. of a white amorphous powder was obtained.

For analysis the substance was dried *in vacuo* over dehydrite. Found, Ba 40.41, 40.11; P 9.23, 9.47.

While the analytical values agree approximately with the calculated composition of barium glycerophosphate containing 2 molecules of water of crystallization, the substance was not pure glycerophosphate, because after hydrolysis with dilute sulfuric acid the solution was found to contain 26 per cent of reducing sugar calculated as glucose. The small amount of available material prevented the identification of the sugar but it is very probable

that the substance was identical with the organic phosphoric acid obtained from the polysaccharide after treatment with hot alcoholic potassium hydroxide, as will be described later.

*Isolation of the Polysaccharide*—The filtrate from the neutral lead acetate precipitate was freed from excess lead with hydrogen sulfide and the filtrate was concentrated to a thick syrup. The syrup was dehydrated by grinding under absolute alcohol as described before. The yield was 4.33 gm. of a white powder.

*Attempts to Dephosphorylate the Polysaccharide*—Several experiments were carried out to determine the best procedure by which to dephosphorylate the polysaccharide. Heating the substance with 14 per cent ammonium hydroxide in a sealed tube to 150–155° for 6 hours or to 160° for 7.5 hours led to incomplete removal of the phosphorus, but heating to 170° for 8.5 hours removed all of the phosphorus but no reducing sugars were liberated.

Examination of the reaction products formed in the preliminary experiments showed that inorganic phosphoric acid, an alcohol-insoluble solid polysaccharide, and an alcohol-soluble syrup had been produced. The alcohol-soluble syrup contained glycerol, the latter being identified as glyceryltribenzoate. The results obtained indicated that some glycerol was combined in the polysaccharide.

In order to split off the glycerol-containing substance, 1.9 gm. of the polysaccharide were refluxed for 2 hours with 1 per cent alcoholic potassium hydroxide. The polysaccharide which remained as an insoluble mass was recovered and dissolved in water. The solution was neutralized with acetic acid and neutral lead acetate was added until no further precipitate occurred. The precipitate was filtered off and washed with water.

The polysaccharide was isolated from the filtrate, as will be described later.

*Isolation of the Organic Phosphoric Acid*—The lead salt was decomposed with hydrogen sulfide in the usual manner and the filtrate was concentrated to dryness *in vacuo*. The residue, which was insoluble in alcohol, indicating that it was not glycerophosphoric acid, was dissolved in water and the solution was neutralized with barium hydroxide. The barium salt was precipitated with alcohol and gave 0.55 gm. of a white amorphous powder.



For analysis the substance was dried at 78° *in vacuo* over dehydrite. Found, Ba 40.79, 40.18; P 8.28, 8.36.

The composition is very similar to that of the barium salt described above and the analytical values agree approximately with the calculated composition of a barium salt of the formula,  $C_6H_{16}O_{14}P_2Ba_2$  (684.8). Calculated Ba 40.13, P 9.05, corresponding to an acid having the formula  $C_6H_{20}O_{14}P_2$ . Such an acid would correspond to mannose-glycerol diphosphoric acid. A similar barium salt was isolated in the original study of mannositose (5).

*Hydrolysis of the Barium Salt*—The balance of the barium salt, 0.529 gm., was dissolved in water and the barium was precipitated by adding sufficient sulfuric acid to make a 5 per cent solution. The barium sulfate was removed and the filtrate was refluxed for 3 hours, after which the sulfuric acid was removed quantitatively with barium hydroxide. After filtration the solution was concentrated *in vacuo* to a volume of 25 cc., neutralized with barium hydroxide, and diluted with an equal volume of alcohol but only a slight precipitate occurred, thus indicating the presence of a small amount of unhydrolyzed organic phosphoric acid. The precipitate was filtered off and the solution was freed of a trace of barium with sulfuric acid and concentrated *in vacuo* to a volume of 3 cc. To this solution, which gave a strong reduction when heated with Fehling's solution, were added 0.25 gm. of phenylhydrazine hydrochloride and 0.2 gm. of sodium acetate. Almost immediately a crystalline precipitate began to separate.

After the crystals had been filtered off, the filtrate was heated in a water bath for an hour but no osazone separated, thus indicating that other hexoses such as glucose or fructose were absent.

The crystalline product mentioned above was recrystallized from 60 per cent alcohol. The crystal form, melting point, and mixed melting point were identical with those of mannose phenylhydrazone.

The only cleavage product definitely identified was mannose phenylhydrazone but indication of the presence of an organic phosphoric acid was obtained and the latter may have been glycerophosphoric acid. It is probable that the original product represented a barium salt of a mannose-glycerol diphosphoric acid

but the definite identification of such an acid will be a problem for future investigation.

*Dephosphorylation of the Polysaccharide*—The filtrate from the lead precipitate mentioned above was freed from excess lead by hydrogen sulfide and, after removal of the lead sulfide, the clear solution was concentrated *in vacuo* to a thick syrup. The syrup was dissolved in 15 cc. of 14 per cent ammonium hydroxide and the solution was heated in a sealed tube to 170° for 8.5 hours. The contents of the tube had a slight straw color and a small amount of precipitate had separated. The solution was filtered and concentrated *in vacuo* to remove the ammonia, after which it was diluted with water and an excess of barium hydroxide was added which caused a heavy precipitate of barium phosphate. The latter was filtered off and discarded.

The filtrate after removal of excess barium was concentrated *in vacuo* to a thick syrup. Since it was impossible to induce crystallization, the substance was precipitated by pouring the syrup into absolute alcohol. The product, a white amorphous powder weighing 1.3 gm., was free from phosphorus and it did not contain any reducing sugar. Attempts to crystallize the substance were unsuccessful.

*Acetylation of the Polysaccharide*—The product described above was treated with 25 cc. of pyridine and 6 cc. of acetic anhydride. Practically all of the substance dissolved on standing at room temperature for 3 days. The solution, after it had been concentrated *in vacuo* to about 5 cc., was poured into dilute sulfuric acid, the reaction product was extracted with chloroform, and the extract was washed with water until the washings were neutral to litmus. The acetyl derivative obtained on evaporation of the solvent was found to be easily soluble in the usual organic solvents and it did not crystallize. The concentrated alcoholic solution of the substance on being mixed with water gave a white amorphous powder which weighed 1.4 gm.

The melting point was not sharp. When heated in a capillary tube, it began to sinter at about 98°, became transparent at 108°, and fused at 112°. The melt did not crystallize on cooling but remained as a transparent mass.

*Rotation*—0.1425 gm. of substance dissolved in methyl al-

cohol and diluted to 10 cc. gave in a 1 dm. tube a reading of  $\alpha = +0.694^\circ$ ; hence  $[\alpha]_D^{25} = +48.7^\circ$ .

*Analysis*—

$C_{19}H_{30}O_{16}(\text{COCH}_3)_{12}$ (1008).	Calculated.	C 50.00, H 5.55
	Found.	" 49.48, " 5.97
Mol. wt. (Rast), 907, 983, 940, 966		

*Saponification of the Acetyl Derivative*—Since the acetyl derivative could not be obtained in crystalline form, it was saponified and the free glycoside was isolated. For saponification 1.0 gm. of the acetyl derivative was refluxed for 4 hours with 120 cc. of methyl alcohol and 50 cc. of a saturated aqueous solution of barium hydroxide. The amount of barium hydroxide neutralized during the saponification was equivalent to 11.30 cc. of  $\text{N CH}_3\text{COOH}$ , corresponding to 67.78 per cent of acetic acid.

*Isolation of Manninositose*—After the methyl alcohol had been distilled off under reduced pressure and the barium had been precipitated quantitatively with sulfuric acid, the filtrate was evaporated *in vacuo* to dryness. The residue was a thick syrup which was very soluble in water but insoluble in alcohol and which could not be induced to crystallize. The syrup was dissolved in 10 cc. of water and the solution was poured with constant stirring into 200 cc. of absolute alcohol, whereupon a finely divided precipitate separated. The precipitation was completed by adding 100 cc. of ether, after which the precipitate was collected, washed with absolute alcohol, and dried *in vacuo*. The white amorphous powder weighed 0.45 gm. The substance had no definite melting point. When heated in a capillary tube, it began to fuse at about  $215^\circ$ , showed slight effervescence at about  $250^\circ$ , and turned faintly yellowish in color.

*Rotation*—0.2207 gm. of substance dissolved in water and diluted to 10 cc. gave in a 1 dm. tube a reading of  $\alpha = +1.637^\circ$ ; hence  $[\alpha]_D^{25} = +74.1^\circ$ . The solution showed no mutarotation.

*Hydrolysis of Manninositose*—When the glycoside was refluxed with 5 per cent sulfuric acid, the maximum reduction as determined by the Shaffer-Hartmann method (6) was attained in about 2.5 hours and amounted to 63 per cent calculated as glucose. For the determination of the cleavage products 0.4 gm. of the glycoside was refluxed with 60 cc. of 5 per cent sulfuric

acid for 4 hours, after which the sulfuric acid was removed quantitatively with barium hydroxide and filtered from barium sulfate. The filtrate was concentrated *in vacuo* to a volume of 8 cc.

*Separation of Mannose As Phenylhydrazone*—The solution mentioned above was mixed with 0.4 gm. of phenylhydrazine dissolved in 1.0 cc. of alcohol. The mannose phenylhydrazone began to crystallize almost immediately and after the solution had stood overnight the crystals were filtered off, washed with water and with alcohol, and dried *in vacuo*. The faintly yellow-colored crystals weighed 0.3465 gm., corresponding to 0.2310 gm. of mannose, equivalent to 57.75 per cent of the glycoside. After recrystallization from 60 per cent alcohol large, nearly colorless crystals were obtained which melted when rapidly heated at 194–195° with decomposition and did not depress the melting point of pure mannose phenylhydrazone which melted at the same temperature.

*Isolation of Inosite*—The filtrate from the mannose phenylhydrazone was freed of excess phenylhydrazine by means of benzaldehyde in the usual manner and the excess of benzaldehyde was removed by extraction with chloroform. The solution, after being decolorized with norit and concentrated to about 4.0 cc., was mixed with alcohol until it turned slightly cloudy. On standing, prismatic crystals separated and were collected, washed with alcohol, and dried *in vacuo*. Addition of ether to the mother liquor caused another small crop of crystals to separate which were filtered off, washed, dried, and combined with the first lot. The total yield of crystalline inosite was 0.1105 gm., corresponding to 27.62 per cent of the glycoside.

The mother liquor on evaporation to dryness left a solid residue which weighed about 50 mg. When this material was dissolved in a little water and tested with Fehling's solution, a slight reduction was obtained. The substance yielded neither an insoluble phenylhydrazone nor an osazone on treatment with phenylhydrazine. Undoubtedly some inosite was present because inosite does not separate quantitatively from a solution.

The inosite on recrystallization from water by the addition of alcohol separated in characteristic needle-shaped prisms. It gave the reaction of Scherer, melted at 225°, and caused no depression of the melting point when mixed with pure inosite.

The amounts of the cleavage products obtained show that

about 2 parts of mannose and 1 part of inosite were recovered and the total recovery amounted to 85.37 per cent of the glycoside. These values would indicate that manninositose is a triglycoside containing 2 molecules of mannose combined with 1 molecule of inosite.

#### SUMMARY

The phosphatide of the human tubercle bacillus contains at least two types of carbohydrates and both contain phosphorus in organic combination.

One carbohydrate appears to be a mannose-glycerol diphosphoric acid which gives a water-insoluble lead salt.

The other carbohydrate is a phosphorus-containing glycoside, manninositose phosphoric acid, which is not precipitated from aqueous solution by lead acetate.

Manninositose phosphoric acid on being heated with dilute ammonium hydroxide to 170° yields inorganic phosphoric acid and the glycoside manninositose.

Manninositose on hydrolysis with dilute sulfuric acid gives only mannose and inosite and approximately in the ratio of 2:1.

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# QUANTITATIVE INVESTIGATIONS OF AMINO ACIDS AND PEPTIDES

## IV. THE SOLUBILITIES OF THE AMINO ACIDS IN WATER- ETHYL ALCOHOL MIXTURES\*

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The solubilities of the amino acids in water were reported in a previous paper (2). The present studies on the solubilities of eight amino acids in five concentrations of ethanol at four temperatures were undertaken because only a limited number of values are given in the literature and because dependable solubility data are of both theoretical and practical importance.

### EXPERIMENTAL

*Amino Acids*<sup>1</sup>—The amino acids used in these investigations were prepared in this laboratory. The purity of the samples was insured by repeated crystallization from water or aqueous alcohol and by analysis. Water was used as the solvent for the final crystallization of *dl*-leucine and *dl*-norleucine, 40 per cent ethanol for *dl*-valine, and 70 to 80 per cent ethanol for the other amino acids.

All but three of the amino acid samples were analyzed by titration with standard base by the formaldehyde-glass electrode

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A preliminary report of this work was given before the American Society of Biological Chemists at Memphis, April 21-24, 1937. An abstract of this paper has appeared (1).

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method of Dunn and Loshakoff (3). The milliequivalents of base required to titrate eleven samples of the other five amino acids averaged  $99.93 \pm 0.39$  per cent of the theoretical amount. These analyses were performed during the preliminary studies on the glass electrode method prior to the development of the technique which has been shown to be of high precision and accuracy. Since it has been found repeatedly in later experiments that amino acids purified as described in the present paper are almost invariably analytically pure ( $100.0 \pm 0.1$  per cent), it seems probable that the amino acids used for the solubility studies were of higher purity than is indicated by the data quoted. Additional analyses by the improved technique were not made, since adequate samples of the original amino acids were no longer available.

The solubility of only one optically active amino acid, *d*-glutamic acid, was determined, because in many instances present knowledge of the specific rotations of the amino acids is inadequate to serve as a reliable criterion of purity. The difficulties encountered by Cohn and coworkers (4) with *l*-leucine illustrate this point. The values of  $[\alpha]_D^{25}$  for the two samples of *d*-glutamic acid used in the present work were  $+31.4^\circ$  and  $+32.0^\circ$  when the molal ratio of HCl to amino acid was 1.55:1. Because these figures do not agree as closely as desired with those reported previously (2, 5), experiments are to be undertaken to explain the differences.

*Solvents*—Distilled water which had been in contact only with block tin was used without further purification. It was shown, however, by gravimetric analysis on four 50 ml. samples that the distilled water contained an average of only 0.0002 per cent by weight of non-volatile impurities.

Commercial ethyl alcohol was purified by fractional distillation through a Hempel column. The first and last tenths of each distillate were discarded to eliminate traces of diethyl ether, higher alcohols, and other low or high boiling impurities. Although the purified alcohol was not tested for ether, presumably the latter was removed, because, as shown by McKelvy (6), its presence in alcohol would be readily detected by the strong odor. Higher alcohols were shown to be absent, since 5 ml. of the purified alcohol, treated with 5 ml. of concentrated sulfuric acid, gave no color after standing for 8 days.

Qualitative tests on the purified alcohol with Schiff's sulfite-

fuchsin reagent indicated that aldehyde was present. The content of aldehyde (expressed as acetaldehyde) was found to be only 0.004 per cent by weight when the alcohol was analyzed by a sulfite-fuchsin colorimetric procedure (7) with solutions containing from 0.0001 to 0.0006 gm. by weight of pure acetaldehyde in aldehyde-free ethyl alcohol as comparison standards. The aldehyde-free alcohol was prepared by digesting anhydrous alcohol with metallic aluminum and potassium hydroxide by the method of Stout and Schuette (8) to remove acetaldehyde, since this, the most common impurity, is not entirely removed by fractional distillation according to the observations of McKelvy (6). Since the latter investigator found that the aldehyde content of an aldehyde-free alcohol increased to the original concentration (0.0027 per cent) on exposure to diffused daylight for a few hours or to the dark for a few weeks, the preparation of aldehyde-free alcohol for use in the solubility measurements was considered to be impractical. It was assumed, moreover, that traces of acetaldehyde could have no measurable influence on the amino acid solubility values.

Because it was evident that the quantities of non-volatile material in the alcoholic solvents must be negligible and definitely known if the solubility values, especially those in concentrated alcohol, were to have quantitative significance, twenty analyses were made on the various solvents during the course of the experiments. In each determination a 50 ml. sample of the solvent was evaporated to constant weight at 60°. It was found that 0.0006 gm. was the maximum and 0.000019 gm. the average weight of non-volatile residue per 50 ml. of solvent.

In view of the intention at the beginning of these studies to measure amino acid solubilities in anhydrous ethyl alcohol, the latter was prepared by treating commercial ethyl alcohol with calcium oxide. The purity of the alcohol was determined by measuring its miscibility temperature with a standard paraffin oil-kerosene mixture by the Crismer method as modified by Andrews (9).

A few determinations were made of the solubilities of amino acids in anhydrous ethyl alcohol, but this work was discontinued because the acetaldehyde, which formed when the alcohol stood for some time in the dark, gave colored reaction products with the



amino acids during the evaporation of the solutions for gravimetric analysis. The use of anhydrous alcohol was abandoned, also, because the solubilities of the amino acids in this solvent were too low to be determined with high accuracy by the authors' method.

*Method of Analysis*—The densities of the alcoholic solvents were determined to an accuracy of  $\pm 0.0001$  by means of a Westphal balance which had been calibrated against purified distilled water.

The quantities of amino acids dissolved in the alcoholic solvents were determined by a gravimetric method essentially the same as that described in a previous paper (2). Florence flasks of 50, 200, and 250 ml. capacity to which 5 inch lengths of 12 mm. Pyrex tubing had been sealed at the necks were used as containers. These special flasks were cleaned with cleaning solution, rinsed ten times with tap water, ten times with distilled water, and twice with the alcoholic solution in the order given.

The purified amino acid and the solvent were placed in duplicate flasks of appropriate size which were sealed immediately by fusing the necks with an oxygen flame. One of the mixtures was brought to a temperature about  $10^\circ$  above, and the other about  $10^\circ$  below, that of the thermostat. For the determinations at  $0^\circ$  one mixture was cooled to about  $-5^\circ$ . Each mixture was maintained at the specified temperature for about 15 minutes and was shaken at intervals during this time to insure the formation of a nearly saturated solution. As noted previously by Zittle and Schmidt (10) a somewhat longer time is required to prepare saturated solutions of *dl*-leucine, *dl*-norleucine, and *dl*-valine than the other amino acids investigated, because of the difficulty with which they are wetted by an aqueous solvent. Since the flasks were sealed prior to the described treatment, the times required for the mixtures to attain the desired temperatures were determined by preliminary standardization experiments with open flasks. Each flask was clamped in the thermostat in an upright position with the labeled portion of the neck emerging from the water and shaken by hand at hourly intervals for 8 hours during the day in such a manner that none of the mixture came into the neck of the flask at any time. This precaution was taken to avoid possible decomposition of the amino acid when the flask was opened by breaking the tip of the neck with an electrically heated wire.

Calibrated 5, 10, 25, and 50 ml. pipettes were used to remove aliquots. At 45° and 65° the pipettes were previously heated to the desired temperatures by suspending them in long tubes immersed in the thermostat. The samples were pipetted as quickly as possible through medicinal cotton or both cotton and silk filters. The latter were required to remove completely the suspensions of fine crystals which were present in 75 per cent ethanol solutions of *d*-glutamic and *dl*-aspartic acids at 0°. Solubility determinations of these amino acids in 95 per cent ethanol at 0° could not be made, because the solutions could not be freed from suspended material by filtration.

Five aliquots were taken from the duplicate flasks. One of the aliquots was weighed and the density of the sample determined from its weight and volume. The probable error in some of the density values was about 0.2 per cent, but in others was as high as 0.5 per cent because of the unavoidable slight cooling or heating of the pipettes during the removal of samples from the thermostat. Two aliquots from each of the duplicate flasks were placed in crystallizing dishes of approximately 30 gm. weight and 80 ml. capacity and evaporated to constant weight at 60° in an oven heated by 50 volts a.c. A low voltage current was used to prevent possible ignition of the alcohol vapors. All weighings, except those for the determination of density, were made with a new chainomatic balance.

A water thermostat was employed at 25°, 45°, and 65° and an ice-water mixture at 0°. At 25° and 45° the water level was maintained by an automatic device and at 65° the rate of evaporation was minimized by a layer of paraffin which is liquid at this temperature. A Beckmann thermometer and a Central Scientific Company 50° thermometer, graduated in 0.1°, were calibrated against a Bureau of Standards thermometer and used in measuring the thermostat temperatures. The temperatures of the thermostats, normally regulated to about 0.05°, are given in Table I.

The times found to be adequate for the attainment of equilibrium ranged from 26 hours for *dl*-norleucine in 50 per cent ethanol at 45° to 246 hours for *dl*-alanine in 25 per cent ethanol at 45°. The equilibrium times for all of the amino acids in the several alcohol solutions ranged from 108 to 154 (average, 125) hours at 0°, 129 to 157 (average, 147) hours at 25°, 107 to 128 (average,

TABLE I  
Summary of Experimental Data and Calculations

	Ethanol		Temperature of thermostat*	Density of aliquot of saturated solution (hot or cold side)	Solute per 100 gm. solvent, corrected†	N (mole fraction solute)	Solute in aliquot (average of hot and cold sides), corrected	Dielectric constant	
	Weight	Volume at 20°						D (solvent)	D' (solution)
	per cent	per cent							
<i>Dl</i> -Alanine‡	20.32	24.93	0.00 ± 0.00	0.979	3.84	$0.878 \times 10^{-2}$	$4.05 \times 10^{-1}$	75.70	84.89
	42.52	50.10	0.00 ± 0.00	0.934	1.16	$0.315 \times 10^{-2}$	$1.19 \times 10^{-1}$	61.25	63.95
	66.94	74.50	0.00 ± 0.00	0.880	0.305	$0.104 \times 10^{-2}$	$0.299 \times 10^{-1}$	45.85	46.53
	92.54	95.14	0.00 ± 0.00	0.817	0.0167	$0.772 \times 10^{-4}$	$0.15 \times 10^{-2}$	31.70	31.73
	20.32	24.93	24.97 ± 0.05	0.984	7.09	$1.61 \times 10^{-2}$	$7.31 \times 10^{-1}$	66.70	83.29
	42.52	50.10	24.97 ± 0.05	0.929	2.52	$0.681 \times 10^{-2}$	$2.56 \times 10^{-1}$	53.45	59.26
	66.94	74.20	24.97 ± 0.05	0.868	0.573	$0.195 \times 10^{-2}$	$5.55 \times 10^{-2}$	39.60	40.86
	92.61	95.14	25.09 ± 0.05	0.807	0.0329	$0.152 \times 10^{-3}$	$2.97 \times 10^{-3}$	26.90	26.97
	20.62	25.28	45.16 ± 0.06	0.989	10.6	$0.239 \times 10^{-2}$	$10.6 \times 10^{-1}$	60.25	84.31
	42.52	50.10	44.96 ± 0.03	0.928	4.25	$0.114 \times 10^{-1}$	$4.24 \times 10^{-1}$	48.00	57.62
	66.94	74.20	44.98 ± 0.03	0.856	0.949	$0.321 \times 10^{-2}$	$0.903 \times 10^{-1}$	35.10	37.15
	92.61	95.14	45.19 ± 0.03	0.794	0.0545	$0.252 \times 10^{-3}$	$0.484 \times 10^{-2}$	23.80	23.91
	20.32	24.93	64.96 ± 0.06	0.994	15.9	$0.353 \times 10^{-1}$	$15.3 \times 10^{-1}$	54.75	89.48
	42.52	50.10	64.94 ± 0.10	0.922	6.68	$0.179 \times 10^{-1}$	$6.47 \times 10^{-1}$	43.20	57.89
	66.94	74.20	64.94 ± 0.10	0.847	1.48	$0.502 \times 10^{-2}$	$13.9 \times 10^{-2}$	31.15	34.31
	92.54	95.09	65.15 ± 0.10	0.780	0.0851	$0.393 \times 10^{-3}$	$7.45 \times 10^{-3}$	21.10	21.27

dL-Asp	dS	dL-Asp										d-Glu									
		20.	24	± 0.03	0.972	0.0703	1.09 × 10 <sup>-4</sup>	5.12 × 10 <sup>-3</sup>	75	82	20.	24	± 0.03	0.972	0.0703	1.09 × 10 <sup>-4</sup>	5.12 × 10 <sup>-3</sup>	75	82		
42.	50	± 0.03	0.935	0.0267	0.489 × 10 <sup>-4</sup>	1.87 × 10 <sup>-3</sup>	61	29	66.	74	± 0.03	0.886	0.0111	0.253 × 10 <sup>-4</sup>	0.74 × 10 <sup>-3</sup>	45	87				
20.	24	± 0.06	0.963	0.266	0.410 × 10 <sup>-3</sup>	1.91 × 10 <sup>-3</sup>	66	42	20.	24	± 0.06	0.926	0.0992	1.81 × 10 <sup>-4</sup>	0.689 × 10 <sup>-3</sup>	53	56				
42.	50	± 0.06	0.872	0.0317	0.724 × 10 <sup>-4</sup>	2.07 × 10 <sup>-3</sup>	39	63	67.	74	± 0.05	0.807	0.0020	0.62 × 10 <sup>-5</sup>	1.2 × 10 <sup>-4</sup>	26	90				
20.	24	± 0.12	0.958	0	1.05 × 10 <sup>-3</sup>	4.86 × 10 <sup>-3</sup>	60	55	20.	24	± 0.04	0.914	0	0.467 × 10 <sup>-3</sup>	1.75 × 10 <sup>-3</sup>	47	95				
42.	50	± 0.04	0.914	0	1.39 × 10 <sup>-4</sup>	3.90 × 10 <sup>-3</sup>	35	35	67.	74	± 0.02	0.856	0	0.13 × 10 <sup>-4</sup>	2.6 × 10 <sup>-4</sup>	23	80				
67.	74	± 0.03	0.796	0	2.36 × 10 <sup>-3</sup>	1.~ × 10 <sup>-1</sup>	54	75	92.	95	± 0.03	0.952	1.53	2.36 × 10 <sup>-3</sup>	1.~ × 10 <sup>-1</sup>	54	75				
92.	95	± 0.03	0.952	0.581	1.07 × 10 <sup>-3</sup>	3. × 10 <sup>-3</sup>	43	20	32.	32	± 0.03	0.902	0.581	1.07 × 10 <sup>-3</sup>	3. × 10 <sup>-3</sup>	43	20				
32.	32	± 0.03	0.902	0.132	0.302 × 10 <sup>-3</sup>	0. × 10 <sup>-3</sup>	31	15	52.	50	± 0.03	0.840	0.132	0.302 × 10 <sup>-3</sup>	0. × 10 <sup>-3</sup>	31	15				
94.	74	± 0.03	0.840	0.012	0.400 × 10 <sup>-4</sup>	0. × 10 <sup>-3</sup>	21	05	94.	74	± 0.03	0.780	0.012	0.400 × 10 <sup>-4</sup>	0. × 10 <sup>-3</sup>	21	05				
61	95	± 0.03	0.780	0.085	1.19 × 10 <sup>-4</sup>	5. × 10 <sup>-3</sup>	75	74	61	95	± 0.04	0.971	0.085	1.19 × 10 <sup>-4</sup>	5. × 10 <sup>-3</sup>	75	74				
16	24	± 0.04	0.971	0.037	0.613 × 10 <sup>-4</sup>	2. × 10 <sup>-3</sup>	61	--	16	24	± 0.04	0.971	0.037	0.613 × 10 <sup>-4</sup>	2. × 10 <sup>-3</sup>	61	--				
59	50	± 0.09	0.934	0	0.338 × 10 <sup>-4</sup>	0. × 10 <sup>-3</sup>	45	83	59	50	± 0.09	0.934	0	0.338 × 10 <sup>-4</sup>	0. × 10 <sup>-3</sup>	45	83				
03	74	± 0.03	0.882	0.016	0.338 × 10 <sup>-4</sup>	0. × 10 <sup>-3</sup>	45	83	03	74	± 0.03	0.882	0.016	0.338 × 10 <sup>-4</sup>	0. × 10 <sup>-3</sup>	45	83				
00	24	± 0.09	0.964	0.292	4.08 × 10 <sup>-4</sup>	19. × 10 <sup>-3</sup>	66	99	00	24	± 0.09	0.964	0.292	4.08 × 10 <sup>-4</sup>	19. × 10 <sup>-3</sup>	66	99				
66	50	± 0.11	0.925	0.131	2.17 × 10 <sup>-4</sup>	8. × 10 <sup>-3</sup>	53	40	66	50	± 0.11	0.925	0.131	2.17 × 10 <sup>-4</sup>	8. × 10 <sup>-3</sup>	53	40				
11	74	± 0.05	0.867	0.037	7.68 × 10 <sup>-5</sup>	2. × 10 <sup>-3</sup>	39	55	11	74	± 0.05	0.867	0.037	7.68 × 10 <sup>-5</sup>	2. × 10 <sup>-3</sup>	39	55				
61	95	± 0.05	0.807	0.004	1.2 × 10 <sup>-5</sup>	0. × 10 <sup>-3</sup>	26	90	61	95	± 0.05	0.807	0.004	1.2 × 10 <sup>-5</sup>	0. × 10 <sup>-3</sup>	26	90				
0	100	± 0.05	0.783	0.002	7.8 × 10 <sup>-6</sup>	0. × 10 <sup>-3</sup>	24	25	0	100	± 0.05	0.783	0.002	7.8 × 10 <sup>-6</sup>	0. × 10 <sup>-3</sup>	24	25				
00	24	± 0.03	0.960	0.811	1.13 × 10 <sup>-3</sup>	52. × 10 <sup>-3</sup>	60	70	00	24	± 0.03	0.960	0.811	1.13 × 10 <sup>-3</sup>	52. × 10 <sup>-3</sup>	60	70				
59	50	± 0.03	0.912	0.378	6.25 × 10 <sup>-4</sup>	23. × 10 <sup>-3</sup>	47	95	59	50	± 0.03	0.912	0.378	6.25 × 10 <sup>-4</sup>	23. × 10 <sup>-3</sup>	47	95				
11	74	± 0.03	0.854	0.0885	1.83 × 10 <sup>-4</sup>	5. × 10 <sup>-3</sup>	35	05	11	74	± 0.03	0.854	0.0885	1.83 × 10 <sup>-4</sup>	5. × 10 <sup>-3</sup>	35	05				
61	95	± 0.07	0.794	0.0127	3.56 × 10 <sup>-5</sup>	0. × 10 <sup>-3</sup>	23	80	61	95	± 0.07	0.794	0.0127	3.56 × 10 <sup>-5</sup>	0. × 10 <sup>-3</sup>	23	80				
0	100	± 0.03	0.770	0	1.8 × 10 <sup>-5</sup>	0. × 10 <sup>-3</sup>	21	47	0	100	± 0.03	0.770	0	1.8 × 10 <sup>-5</sup>	0. × 10 <sup>-3</sup>	21	47				

***dl*-Leucine**



Ethanol		Temperature of thermostat <sup>a</sup> °C.	Density of aliquot of saturated solution (hot or cold side)	Solute per 100 gm. solvent, corrected <sup>†</sup> gm.	N (mole fraction solute)	Solute in aliquot (average of hot and cold sides) corrected $M_2$ (moles per L.)	Dielectric constant	
Weight	Volume at 20°						D (solvent)	D' (solution)
per cent	per cent							
20.32	24.93	0.00 ± 0.00	0.975	0.530	$1.03 \times 10^{-3}$	$4.89 \times 10^{-2}$	75.70	76.81
42.52	50.10	0.00 ± 0.00	0.938	0.146	$3.35 \times 10^{-4}$	$1.30 \times 10^{-2}$	61.25	61.55
67.27	74.50	0.00 ± 0.00	0.882	0.0304	$0.882 \times 10^{-4}$	$2.55 \times 10^{-3}$	45.75	45.81
92.61	95.14	0.00 ± 0.00	0.818	0.0008	$3 \times 10^{-5}$	$6 \times 10^{-5}$	31.65	31.65
20.32	24.93	25.14 ± 0.05	0.970	1.54	$2.99 \times 10^{-3}$	$1.39 \times 10^{-1}$	66.70	69.85
42.52	50.10	25.14 ± 0.05	0.923	0.461	$1.06 \times 10^{-3}$	$4.02 \times 10^{-2}$	53.45	54.36
67.27	74.50	25.10 ± 0.05	0.869	0.0840	$2.43 \times 10^{-4}$	$6.93 \times 10^{-3}$	39.35	39.51
92.61	95.14	25.09 ± 0.05	0.806	0.0028	$1.1 \times 10^{-5}$	$2.15 \times 10^{-4}$	26.90	26.90
20.32	24.93	45.15 ± 0.12	0.968	3.14	$6.09 \times 10^{-3}$	$2.79 \times 10^{-1}$	60.40	66.73
42.52	50.10	45.04 ± 0.03	0.912	0.935	$2.27 \times 10^{-3}$	$8.46 \times 10^{-2}$	48.00	49.92
66.94	74.20	45.04 ± 0.03	0.857	0.185	$5.35 \times 10^{-4}$	$1.50 \times 10^{-2}$	35.10	35.44
92.61	95.14	45.18 ± 0.03	0.793	0.0058	$2.3 \times 10^{-5}$	$4.38 \times 10^{-4}$	23.80	23.81
20.32	24.93	65.26 ± 0.03	0.974	5.99	$1.15 \times 10^{-3}$	$5.24 \times 10^{-1}$	54.75	66.64
42.52	50.10	65.25 ± 0.03	0.909	1.88	$4.31 \times 10^{-3}$	$1.59 \times 10^{-1}$	43.20	46.80
67.27	74.50	65.24 ± 0.03	0.844	0.318	$9.21 \times 10^{-4}$	$2.53 \times 10^{-2}$	30.95	31.52
92.61	95.14	65.01 ± 0.03	0.783	0.0152	$5.94 \times 10^{-5}$	$1.1 \times 10^{-3}$	21.05	21.06
20.32	24.93	0.02 ± 0.03	0.975	2.10	$0.366 \times 10^{-3}$	$1.72 \times 10^{-1}$	75.70	79.60
42.52	50.10	0.02 ± 0.03	0.942	0.769	$0.159 \times 10^{-2}$	$0.612 \times 10^{-1}$	61.25	62.64
66.94	74.20	0.02 ± 0.03	0.881	0.269	$0.695 \times 10^{-3}$	$2.02 \times 10^{-2}$	45.85	46.31
92.61	95.14	0.01 ± 0.01	0.819	0.0277	$0.974 \times 10^{-4}$	$1.94 \times 10^{-3}$	31.65	31.69
100.0	100.0	0.03 ± 0.03	0.797	0.0136	$0.534 \times 10^{-4}$	$0.92 \times 10^{-3}$	28.23	28.25

dl-S

Val

<i>dl</i> -Valine—con- tinued	25.28		24.85 ± 0.04		0.971	3.30	0.575 × 10 <sup>-3</sup>		2.65 × 10 <sup>-1</sup>		66.55	72.57
	43.36	50.99	24.85 ± 0.13	0.924	0.924	1.53	0.317 × 10 <sup>-3</sup>	1.18 × 10 <sup>-1</sup>	1.18 × 10 <sup>-1</sup>	52.80	55.48	
	67.11	74.35	24.93 ± 0.07	0.870	0.870	0.570	0.147 × 10 <sup>-3</sup>	0.419 × 10 <sup>-1</sup>	0.419 × 10 <sup>-1</sup>	39.55	40.50	
	92.61	95.14	25.04 ± 0.05	0.807	0.807	0.0569	0.200 × 10 <sup>-3</sup>	3.8 × 10 <sup>-3</sup>	3.8 × 10 <sup>-3</sup>	26.90	26.99	
	20.00	24.55	44.91 ± 0.03	0.966	0.966	5.10	0.884 × 10 <sup>-3</sup>	3.99 × 10 <sup>-1</sup>	3.99 × 10 <sup>-1</sup>	60.70	69.76	
	42.66	50.25	44.92 ± 0.03	0.918	0.918	2.74	0.564 × 10 <sup>-3</sup>	2.08 × 10 <sup>-1</sup>	2.08 × 10 <sup>-1</sup>	47.90	52.62	
	67.11	74.35	44.92 ± 0.03	0.857	0.857	0.999	0.259 × 10 <sup>-3</sup>	7.24 × 10 <sup>-3</sup>	7.24 × 10 <sup>-3</sup>	35.05	36.69	
	92.61	95.14	45.21 ± 0.03	0.795	0.795	0.0979	0.345 × 10 <sup>-3</sup>	0.663 × 10 <sup>-3</sup>	0.663 × 10 <sup>-3</sup>	23.80	23.95	
	20.00	24.55	65.07 ± 0.06	0.962	0.962	7.44	1.28 × 10 <sup>-3</sup>	5.69 × 10 <sup>-1</sup>	5.69 × 10 <sup>-1</sup>	55.01	67.93	
	42.52	50.10	64.94 ± 0.03	0.905	0.905	4.49	0.922 × 10 <sup>-3</sup>	3.31 × 10 <sup>-1</sup>	3.31 × 10 <sup>-1</sup>	43.20	50.71	
	66.94	74.20	64.94 ± 0.03	0.841	0.841	1.62	0.417 × 10 <sup>-3</sup>	1.14 × 10 <sup>-1</sup>	1.14 × 10 <sup>-1</sup>	31.15	33.74	
	92.54	95.09	65.15 ± 0.05	0.782	0.782	0.167	0.585 × 10 <sup>-3</sup>	1.10 × 10 <sup>-3</sup>	1.10 × 10 <sup>-3</sup>	21.10	21.35	

\* The recorded deviations from the mean temperatures are in all cases the maximum deviations observed.

† The correction referred to is for the weight of non-volatile impurities per 100 gm. of solvent.

‡ The solubility values in 25 per cent ethanol at 45° for *DL*-alanine samples, one crystallized from water and the other from 80 per cent ethanol, were found to be identical. It may be concluded from these results that solubility differences, if any, of different types of *DL*-alanine crystals are very small. It may be a proper assumption, also, that this deduction is valid for other amino acids known to exist in different crystalline forms.

§ The solubility of *DL*-aspartic acid in 95 per cent ethanol at 0° could not be determined accurately because the filtered aliquots invariably contained some of the fine suspension of solute which formed under the experimental conditions.

|| The solubility of *D*-glutamic acid in 95 per cent ethanol at 0° could not be determined accurately because the filtered aliquots invariably contained some of the fine suspension which formed under the experimental conditions. The solubility of this amino acid at 65° was not determined, because it was considered probable from the work of Foreman (11) that the results would not be reliable owing to the partial conversion of glutamic to pyrrolidonecarboxylic acid. Pertzoff (12) reported that the solubility of *D*-glutamic acid was 0.0068 gm. per 100 gm. of anhydrous ethanol at 25°. This value is more than twice that found by the present authors under these conditions and somewhat higher than their figures for the solubility of this amino acid in 100 per cent ethanol at 45° and 95 per cent ethanol at 25°.



114) hours at 45°, and 78 to 88 (average, 83) hours at 65°. Exact minimum times were not determined and they would have little significance because of differences in the physical characteristics of the amino acids, rate of stirring, and other factors. Equilibrium times, especially those at 65°, were made as short as possible by the use of powdered amino acids in order to minimize possible decomposition. Because *dl*-valine, *dl*-norleucine, and *dl*-leucine cannot be readily pulverized, the final purification of these amino acids was made in such a manner that small crystals were formed. Difficulties were encountered with *dl*-alanine, and to a lesser extent with glycine, *dl*-aspartic acid, and *dl*-valine, because of their tendency to form supersaturated solutions.

The precision of the experimental values is indicated by the following data.

(a) *Percentage Deviations from Mean at All Temperatures for Weight of Solute in Duplicate Aliquots of All Amino Acids*—25 per cent ethanol, maximum, 0.60 per cent; average (hot side), 0.11 per cent; average (cold side), 0.15 per cent. 50 per cent ethanol, maximum, 1.7 per cent; average (hot side), 0.23 per cent; average (cold side), 0.21 per cent. 75 per cent ethanol, maximum, 3.5 per cent; average (hot side), 0.40 per cent; average (cold side), 0.28 per cent. 95 per cent ethanol, maximum, 5.6 per cent; average (hot side), 1.5 per cent; average (cold side), 2.5 per cent. The indicated high percentage deviations in 95 per cent ethanol occurred because of difficulties in filtering the suspensions of fine crystals or because of unavoidable errors in determining the low weights of solutes.

(b) *Percentage Deviations from Mean at All Temperatures for Average Weight of Solute in Duplicate Aliquots from Hot and Cold Sides*—25 per cent ethanol, maximum, 2.4 per cent; average, 0.78 per cent. 50 per cent ethanol, maximum, 3.1 per cent; average, 0.47 per cent. 75 per cent ethanol, maximum, 2.2 per cent; average, 0.43 per cent. 95 per cent ethanol, maximum, 7.6 per cent; average, 2.0 per cent. These deviations from mean values approximate those calculated previously for water solubilities (2).

The concurrence of the authors' values for glycine, *dl*-alanine, *dl*-valine, *dl*-leucine, and *dl*-norleucine in 0 to 95 per cent ethanol at 25° with those of Holleman and Antusch (13) and Cohn and co-

workers (4) is considered to be further evidence of their dependability. Although the solubility data for *dl*-alanine given by Holleman and Antusch appear to be less satisfactory at higher than at lower temperatures, the values reported by Cohn and co-workers are nearly identical in all cases with those of the present authors. The solubility data reported by other authors for glycine (14, 15), *dl*-alanine (16), *dl*-leucine (14, 17), and *d*-glutamic acid (12, 18) are believed to be less accurate than those considered above.

The relatively low precision attained with the less soluble amino acids at high concentrations of ethanol was expected because of the unavoidable limitations of the authors' gravimetric method. While the figures reported are reliable within the specified limits, additional studies with a more sensitive method of analysis are required to give the absolute values in these cases.

The solubility data for the eight amino acids at 0°, 25°, 45°, and 65° in 25, 50, 75, 95, and 100 per cent ethanol by volume are given in Table I. Other experimental data and calculations of theoretical interest are given also. Curves showing the solubilities of the amino acids in gm. per 100 gm. of solvent at 0° and 65° in 0 to 95 per cent ethanol by volume are given in Figs. 1 and 2. Curves showing the solubilities of the amino acids in gm. per 100 gm. of solvent at 0, 25, and 95 per cent ethanol by volume and at temperatures ranging from 0–65° are given in Figs. 3 to 5. The solubilities of *dl*-leucine (19) and *dl*-serine (20) found by Dalton and Schmidt, as well as the solubilities of the other six amino acids in water, previously reported from this laboratory (2), have been correlated with the present alcohol-water data in Figs. 1 to 3. The consistency of all of these data is apparent.

The solubilities of all of the amino acids studied increase markedly with increasing temperature at constant ethanol concentration and decrease similarly with increasing ethanol concentration at constant temperature. In all cases the solubilities are negligible in ethanol solutions above 95 per cent concentration. The pronounced changes in slope undergone by the curves for *dl*-leucine and *dl*-norleucine as the temperature increases from 0–65° (Figs. 1 and 2) are of unusual interest.

As may be seen from Figs. 3 to 5 the relative positions of the

amino acid solubility curves tend to be reversed as the concentration of the ethanol changes from 0 to 95 per cent. The dicarboxylic amino acids, *dl*-aspartic and *d*-glutamic, have intermediate solubility in water but least solubility in 95 per cent ethanol. On the other hand, glycine, *dl*-alanine, and *dl*-serine progressively decrease in relative solubility, while *dl*-valine,

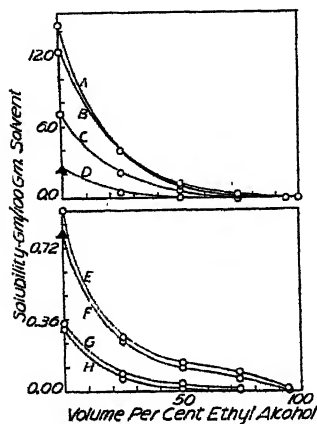


FIG. 1

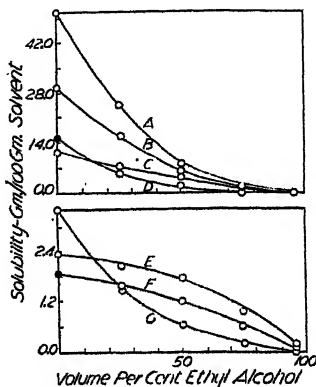


FIG. 2

FIG. 1. Curves showing the solubilities of certain amino acids at 0° in water, ethyl alcohol, and water-ethyl alcohol mixtures. The data are plotted according to the following notation: Curve A, glycine; Curve B, *dl*-alanine; Curve C, *dl*-valine; Curve D, *dl*-serine; Curve E, *dl*-norleucine; Curve F, *dl*-leucine; Curve G, *d*-glutamic acid; Curve H, *dl*-aspartic acid; ▲, Dalton and Schmidt's (19, 20) values.

FIG. 2. Curves showing the solubilities of certain amino acids at 65° in water and water-ethyl alcohol mixtures. The data are plotted according to the following notation: Curve A, glycine; Curve B, *dl*-alanine; Curve C, *dl*-valine; Curve D, *dl*-serine; Curve E, *dl*-norleucine; Curve F, *dl*-leucine; Curve G, *dl*-aspartic acid; ●, Dalton and Schmidt's (19, 20) values.

*dl*-leucine, and *dl*-norleucine become relatively more soluble as the concentration of ethanol increases. The rule that the  $\alpha$ -amino acids are less soluble in ethanol-water mixtures than in water applies only to those with eight or fewer  $\text{CH}_2$  groups, according to observations reported recently by Cohn (21).

The curves shown in Figs. 6 to 8 were obtained by plotting  $\log N$  (mole fraction of the solute) against the reciprocal of the ab-

solute temperature. Since all of the curves are nearly straight lines, it may be concluded that the deviations from perfect solution laws are not of large magnitude. The thermodynamic activities which have been determined for a few amino acids in

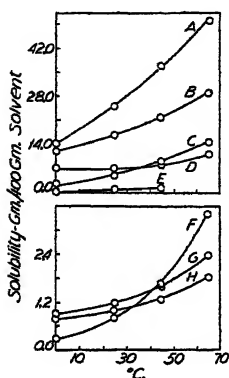


FIG. 3

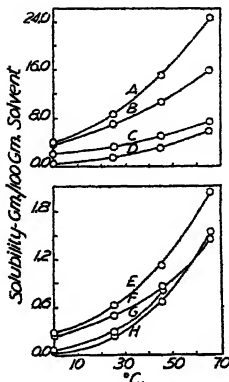


FIG. 4

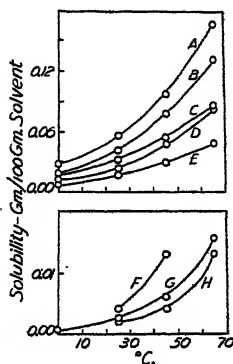


FIG. 5

FIG. 3. Curves showing the solubilities of certain amino acids in water at various temperatures. The data are plotted according to the following notation: Curve A, glycine; Curve B, *dl*-alanine; Curve C, *dl*-serine (Dalton and Schmidt's (20) values); Curve D, *dl*-valine; Curve E, *d*-glutamic acid; Curve F, *dl*-aspartic acid; Curve G, *dl*-norleucine; Curve H, *dl*-leucine (Dalton and Schmidt's (19) values).

FIG. 4. Curves showing the solubilities of certain amino acids at various temperatures in approximately 25 per cent ethyl alcohol by volume. The data are plotted according to the following notation: Curve A, glycine; Curve B, *dl*-alanine; Curve C, *dl*-valine; Curve D, *dl*-serine; Curve E, *dl*-norleucine; Curve F, *dl*-leucine; Curve G, *d*-glutamic acid; and Curve H, *dl*-aspartic acid.

FIG. 5. Curves showing the solubilities of certain amino acids at various temperatures in approximately 95 per cent ethyl alcohol by volume. The data are plotted according to the following notation: Curve A, *dl*-valine; Curve B, *dl*-norleucine; Curve C, *dl*-alanine; Curve D, *dl*-leucine; Curve E, glycine; Curve F, *d*-glutamic acid; Curve G, *dl*-serine; and Curve H, *dl*-aspartic acid.

aqueous solution at 0° and 25° reveal the extent of the abnormalities in these cases, but, unfortunately, the majority of the authors' curves cannot be interpreted because of the lack of activity data. The activity coefficients of glycine in water at 0° and 25° are given

in a recent paper by Smith and Smith (22). It is evident from these data that the activity coefficient of glycine is approximately unity when the concentration of solute is about 0.1 mole per 1000 gm. of solvent, but falls to about 0.73 with a concen-

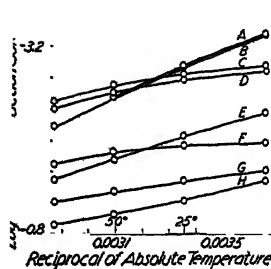


FIG. 6

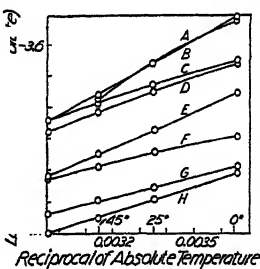


FIG. 7

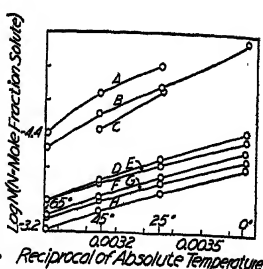


FIG. 8

FIG. 6. Curves showing the relation between the logarithms of the mole fractions of certain amino acids in saturated aqueous solutions and the reciprocals of the absolute temperatures. The data are plotted according to the following notation; Curve A, *d*-glutamic acid; Curve B, *dl*-aspartic acid; Curve C, *dl*-leucine (Dalton and Schmidt's (19) values); Curve D, *dl*-norleucine; Curve E, *dl*-serine (Dalton and Schmidt's (20) values); Curve F, *dl*-valine; Curve G, *dl*-alanine; and Curve H, glycine.

FIG. 7. Curves showing the relation between the logarithms of the mole fractions of certain amino acids in saturated 25 per cent ethyl alcohol by volume and the reciprocals of the absolute temperatures. The data are plotted according to the following notation: Curve A, *d*-glutamic acid; Curve B, *dl*-aspartic acid; Curve C, *dl*-leucine; Curve D, *dl*-norleucine; Curve E, *dl*-serine; Curve F, *dl*-valine; Curve G, *dl*-alanine; and Curve H, glycine.

FIG. 8. Curves showing the relation between the logarithms of the mole fractions of certain amino acids in saturated 95 per cent ethyl alcohol by volume and the reciprocals of the absolute temperatures. The data are plotted according to the following notation: Curve A, *dl*-aspartic acid; Curve B, *dl*-serine; Curve C, *d*-glutamic acid; Curve D, *dl*-leucine; Curve E, glycine; Curve F, *dl*-norleucine; Curve G, *dl*-alanine; and Curve H, *dl*-valine.

tration of 3.3 moles per 1000 gm. of solvent. It is apparent, also, that at 0° the activity coefficients of glycine are smaller than those at 25° by 0.2 to 6.7 per cent in solutions containing from 0.1 to 2.0 moles of solute per 1000 gm. of solvent.

The activity coefficients of glycine in water at 0° and 25° for

the molalities used in calculating the values for  $\log N$  represented by the curve in Fig. 6 were determined from the curves relating activity coefficients and moles of amino acid per 1000 gm. of solvent which were constructed from the data given by Smith and Smith (22). These values,<sup>2</sup> 0.739 at 0° and 0.727 at 25°, and the authors' stoichiometric concentration data were utilized in determining the activities of glycine under these conditions. The  $\log N$  values corresponding to these activities were calculated and plotted against temperature. The straight line connecting these points was extrapolated for use in estimating the activities of glycine for the experimental concentrations measured by the present authors. It was shown by further calculations that the activity coefficients of glycine in water for the experimental concentrations observed at 50° and 75° are approximately 0.742 and 0.795, respectively.

Activity coefficients at 0° for 0.0025 to 0.030 *M* aspartic acid (assumed to be *l*) solutions ranging from 0.202 to 0.051 and for 0.0025 to 0.30 *M* *D*-glutamic acid solutions ranging from 0.169 to 0.44 were reported in 1930 by Hoskins, Randall, and Schmidt (23). Smith and Smith (24) have shown recently that at 25° the activity coefficients for alanine are 1.023 in 1.0 *M* and 1.012 in 0.5 *M* concentrations. At the latter concentration 0.910 was the value reported for serine. These authors found that the activity coefficient curves as a function of molal concentration had widely different slopes, that the activity coefficients increased with increased number of  $\text{CH}_2$  groups in a straight chain, and that the curve for a branched chain amino acid was steeper than that for its normal isomer. In order to interpret the authors' curves (Fig. 6), however, additional data would be required.

The curves for  $\log N$  as a function of temperature at constant alcohol concentration (Figs. 7 and 8) are all very nearly straight lines. It seems probable, therefore, that the activity coefficients as a function of concentration vary approximately linearly under these conditions. That this is the case with dilute aqueous solutions of certain amino acids at comparable concentrations is

<sup>2</sup> The activity coefficient of a saturated aqueous solution of glycine at 25° was assumed to be 0.90 by Zittle and Schmidt (10), although values as low as 0.624 were calculated from the available freezing point data.

evident from the curves plotted with the data of Smith and Smith (22) and Hoskins, Randall, and Schmidt (23).

When  $\log N$  is plotted against volume per cent ethanol at constant temperature (Figs. 9 and 10), deviations of considerable magnitude from straight lines appear, particularly at high concentrations of alcohol. In order to obtain straight lines in these

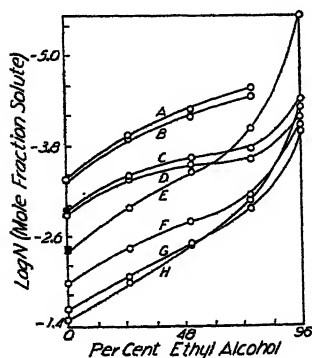


FIG. 9

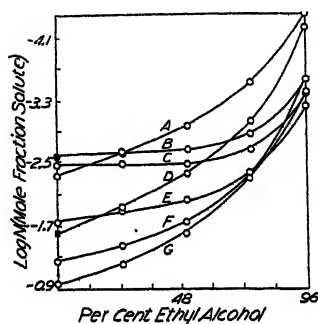


FIG. 10

FIG. 9. Curves showing the relation at 0° between the logarithms of the mole fractions of certain amino acids in saturated water-ethyl alcohol solutions and the per cent ethyl alcohol by volume. The data are plotted according to the following notation: Curve A, *dl*-aspartic acid; Curve B, *d*-glutamic acid; Curve C, *dl*-leucine; Curve D, *dl*-norleucine; Curve E, *dl*-serine; Curve F, *dl*-valine; Curve G, *dl*-alanine; Curve H, glycine; ●, Dalton and Schmidt's (19) value; and ■, Dalton and Schmidt's (20) value.

FIG. 10. Curves showing the relation at 65° between the logarithms of the mole fractions of certain amino acids in saturated water-ethyl alcohol solutions and the per cent ethyl alcohol by volume. The data are plotted according to the following notation: Curve A, *dl*-aspartic acid; Curve B, *dl*-leucine; Curve C, *dl*-norleucine; Curve D, *dl*-serine; Curve E, *dl*-valine; Curve F, *dl*-alanine; Curve G, glycine; ●, Dalton and Schmidt's (19) value; and ■, Dalton and Schmidt's (20) value.

cases the activity coefficients must necessarily increase to exceedingly large values with increasing ethanol concentration. As shown by Scatchard and Prentiss (25) the osmotic coefficients of glycine are larger than 1 and approach unity at 0 M concentration of ethanol. It is particularly significant that the slopes of the curves become more steep at constant molal concentration

of the solute as the proportion of alcohol in the solution increases. It might be expected, therefore, that the activity coefficients, as well as the osmotic coefficients, would increase to very high values even at the low molal concentrations of solute present in solution under these conditions.

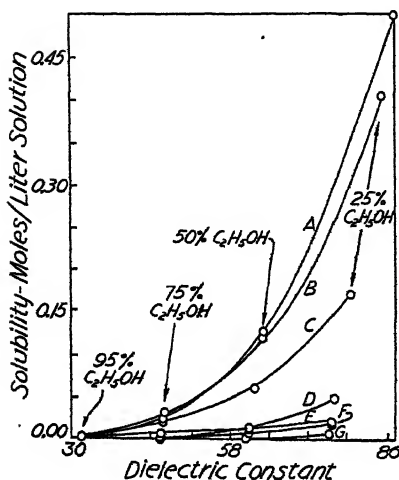


FIG. 11

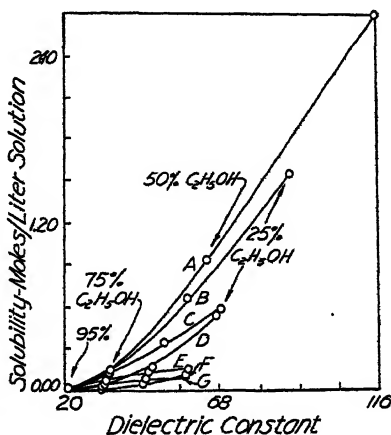


FIG. 12

FIG. 11. Curves showing the relation at 0° between the moles per liter of solution of certain amino acids in saturated water-ethyl alcohol solutions and the dielectric constants of the solutions. The data are plotted according to the following notation: Curve A, glycine; Curve B, *dl*-alanine; Curve C, *dl*-valine; Curve D, *dl*-serine; Curve E, *dl*-norleucine; Curve F, *dl*-leucine; and Curve G, *d*-glutamic and *dl*-aspartic acids.

FIG. 12. Curves showing the relation at 85° between the moles per liter of solution of certain amino acids in saturated water-ethyl alcohol solutions and the dielectric constants of the solutions. The data are plotted according to the following notation: Curve A, glycine; Curve B, *dl*-alanine; Curve C, *dl*-valine; Curve D, *dl*-serine; Curve E, *dl*-norleucine; Curve F, *dl*-aspartic acid; and Curve G, *dl*-leucine.

Curves showing the relation between moles of amino acids per liter of saturated solution and the dielectric constants of these saturated solutions at constant alcohol concentrations are given in Figs. 11 and 12. Similar curves at constant temperature are given in Figs. 13 and 14. The dielectric constants of the authors'



alcohol solvents were calculated from the equation,  $\log D = \log a + b(t - 20)$ , given by Åkerlöf (26), where  $D$  is the dielectric constant of the pure solvent,  $a$  and  $b$  empirical constants, and  $t$  the observed centigrade temperature. The values for  $D$  at  $0^\circ$  and  $65^\circ$  in 0 to 100 per cent ethanol were calculated by means of

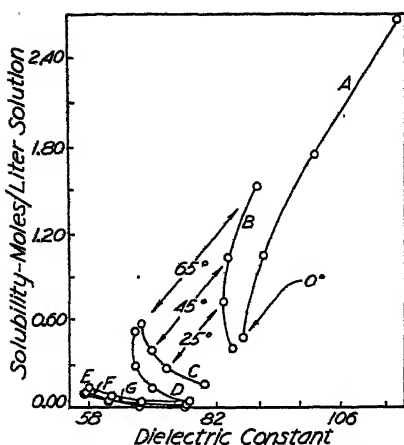


FIG. 13

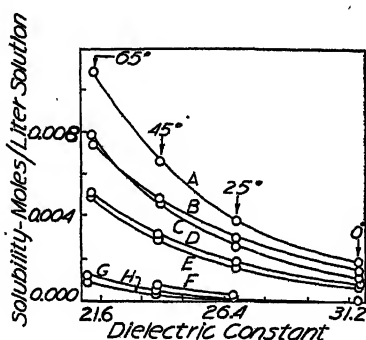


FIG. 14

FIG. 13. Curves showing the relation at various temperatures between the moles per liter of solution of certain amino acids in saturated 25 per cent ethyl alcohol by volume and the dielectric constants of the solutions. The data are plotted according to the following notation: Curve A, glycine; Curve B, *dl*-alanine; Curve C, *dl*-valine; Curve D, *dl*-serine; Curve E, *dl*-norleucine; Curve F, *d*-glutamic and *dl*-aspartic acids; and Curve G, *dl*-leucine.

FIG. 14. Curves showing the relation at different temperatures between the moles per liter of solution of certain amino acids in saturated 95 per cent ethyl alcohol by volume and the dielectric constants of the solutions. The data are plotted according to the following notation: Curve A, *dl*-valine; Curve B, *dl*-alanine; Curve C, *dl*-norleucine; Curve D, glycine; Curve E, *dl*-leucine; Curve F, *d*-glutamic acid; Curve G, *dl*-serine; and Curve H, *dl*-aspartic acid.

this equation and plotted against temperature. The curve drawn through these points was utilized in interpolating the values for  $D$  at the precise volumes per cent of ethanol used experimentally.

The increment in dielectric constant per mole of solute,  $\delta$ , was assumed to be the average value, 22.7, which is the figure given by

Cohn and coworkers (4) as an approximation for  $\alpha$ -amino acids. The dielectric constants for the saturated solutions of the amino acids were calculated from the equation,  $D' = D + \delta M$ , given by Cohn and coworkers (4), where  $D'$  is the dielectric constant of the solution,  $D$  the dielectric constant of the pure solvent,  $\delta$  the increment in dielectric constant per mole of solute, and  $M$  the moles of solute per liter of solution.

It is evident from a consideration of the data given by Wyman and McMeekin (27) that the values for  $\delta$  decrease appreciably with increasing concentrations of ethanol. When the  $\delta$  values for glycine (presumably at 25°) in 0 to 60 per cent ethanol are plotted against per cent ethanol, it is apparent that  $\delta$  becomes approximately 18.6 in 95 per cent ethanol if the curve connecting these points is assumed to be a straight line beyond about 30 per cent ethanol and is extrapolated. The use of the average figure, 22.7, for  $\delta$  in the present calculations introduces only a slight error, since  $M$ , and hence  $\delta M$ , decreases rapidly with increasing ethanol concentrations to values of negligible magnitude in 95 per cent ethanol.

As shown in Figs. 11 to 14, the dielectric constant curves of the saturated solutions are linear only over a limited range of alcohol concentration at constant temperature or of temperature at constant alcohol concentration. The dielectric constants of the 95 per cent ethanol solutions are nearly the same as those of the pure solvents, if the assumption that  $\delta$  is constant is correct. In solutions of lower ethanol concentration the rate of decrease of the dielectric constants is less than in 95 per cent ethanol owing to the rapidly increasing solubilities of the amino acids. In solutions of 50 per cent and lower concentrations of ethanol the slopes of some of the curves are altered so that the natural tendency of increasing temperature to diminish the dielectric constants of the solvents is entirely overcome by the greater influence of the solute in increasing the dielectric constants of the solutions. If, as stated by Wyman and McMeekin (27), small changes in  $\delta$  indicate that the zwitter ion predominates even in solutions of high ethanol concentration, the marked alterations of the dielectric constants of the solutions would be due to gross changes in the quantities of zwitter ions rather than in the proportion of zwitter ions to un-ionized molecules. On the other hand, Edsall and

Blanchard state (28) that the concentration ratio of zwitter ions to uncharged molecules decreases, as the dielectric constant of the solvent is lowered, to ratios of about 500:1 in 90 per cent ethanol to 1:1 in pure alcohol.

In recent studies by Lindquist and Schmidt (29) it was shown that the dielectric constants of aqueous solutions of glycine, alanine, and proline increase linearly with respect to the concentration, expressed as moles per liter of solution, while the molal dielectric increment,  $\delta$ , of glycine and alanine yielded straight lines over the temperature range of from 1–30° when plotted against the temperature. The conclusion of these authors that micellation of the three amino acids is less than 5 per cent is in disagreement with interpretations which have been made of freezing point data.

The present results and conclusions are essentially in agreement with those of Lindquist and Schmidt, although it is evident that the linearity over the temperature range employed by these authors is only approximate for alcohol solutions and that pronounced deviations from straight lines appear when temperatures from 0–65° were investigated. If it is assumed that the dielectric constants of the saturated solutions are linear functions of the molalities at constant temperature for the entire range of alcohol concentrations, this would mean that the values for  $\delta$  must be large for solutions of high percentage alcohol. Since Wyman and McMeekin (27) have found that  $\delta$  decreases from 22.58 in water to 20.56 in 60 per cent ethanol, the curve plotted from these data would have a minimum at some concentration of ethanol above 60 per cent.

#### SUMMARY

1. The solubilities of eight amino acids in 25, 50, 75, and 95 per cent ethanol at 0°, 25°, 45°, and 65° have been determined. Solubilities for *d*-glutamic acid in 100 per cent ethanol at 25° and 45° and for *DL*-valine in 100 per cent ethanol at 0° were also measured.

2. Curves relating solubilities in gm. per 100 gm. of solvent as functions of ethanol concentration at constant temperature have been constructed from the authors' experimental data for the solubilities of amino acids in water and water-ethanol mixtures

and the most reliable data reported in the literature by other workers. Similar curves have been drawn relating solubilities in gm. per 100 gm. of solvent as functions of temperature at constant ethanol concentration.

3. Curves have been drawn relating  $\log N$  (mole fraction of the solute) as a function of temperature at constant ethanol concentration and as a function of ethanol concentration at constant temperature. Similar curves have been drawn relating solubility in moles per liter of solution to the dielectric constants of the solutions at constant temperature and at constant ethanol concentration.

4. The amino acids for which the logarithmic curves were approximately straight lines were assumed to behave more nearly as perfect solutes than those for which greater deviations were noted. The relation of the observed abnormalities to the probable activity coefficients of the solutes investigated was discussed.

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# SOME OBSERVATIONS ON THE CONSTITUTION OF THE "PREGNANETRIOL" OCCURRING IN THE URINE OF PREGNANT MARES

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Some years ago the isolation of a digitonin-non-precipitable, saturated solid alcohol of the formula  $(C_7H_{12}O)_n$  from the neutral, ether-soluble fraction of pregnant mare urine was reported by one of the present authors in collaboration with others (1, 2). It was tentatively suggested that this substance might be a trihydroxy compound closely related to pregnanediol, but since it was not found possible to determine the molecular weight of the substance or that of any one of its derivatives, and since no decomposition products of the substance could be characterized, no definite conclusions concerning its chemical nature could be arrived at.

Marker *et al.* (3) have recently reported the isolation of this substance from the urine of pregnant mares, together with an isomeric compound. The latter compound has been designated by them as "pregnanetriol A" and the former as "pregnanetriol B," although no molecular weight determinations were reported and no evidence of the presence of a pregnane skeleton in either compound was presented. More recently the same workers (4) have presented evidence which they claim proves that pregnanetriol B is pregnane-3( $\alpha$ ),4( $\beta$ ),20-triol.<sup>1</sup> This conclusion is quite irreconcilable with the experimental evidence obtained over a period of several years by the present authors. This evidence, although not yet complete, is presented at this time, since, owing to lack of material, further progress in the near future may be considerably delayed.

<sup>1</sup> The authors wish to express their appreciation of the courtesy of Dr. Oliver Kamm in allowing them to see a proof of this paper, and in discussing with one of them (G. F. M.) the problem of the structure of this compound.

The crystalline material used in this investigation was isolated by a method slightly modified from that originally described.<sup>2</sup>

At the outset of this research the working hypothesis was adopted that the substance was a pregnanetriol or allopregnanetriol, and the possibility was entertained that it might be a 3( $\alpha$ ),4,20-triol formed by the reduction of the two ketonic groups of progesterone and the addition of the elements of water to the double linkage. The complete stability of the compound to treatment with lead tetraacetate, which has been reported by Marker *et al.* (4), and which has also been demonstrated in the present work, seems to prove definitely that a *cis*- $\alpha,\beta$ -glycol grouping cannot be present. The fact that the substance is also completely stable to lead tetraacetate at 37° would suggest that *trans*- $\alpha,\beta$ -glycol grouping cannot be present. The observation made by Marker *et al.* (4) that the compound is unattacked by periodic acid also seems to exclude the possibility of the presence of the latter grouping, although these authors did not so interpret it.

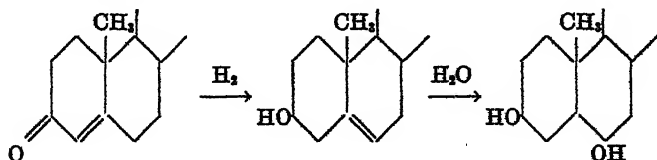
With the ultimate object of preparing the parent saturated hydrocarbon of the compound for comparison with pregnane and allopregnane, an attempt was made to eliminate the hydroxyl groups one at a time by partial hydrolysis of the acetate, oxidation with chromic anhydride, and reduction by the Wolff-Kishner method. Treatment of the acetate with 0.8 *equivalent* of methanolic potassium hydroxide yielded a product melting at 222–224° which, on the assumption that the original compound was indeed a triol of the formula  $C_{21}H_{36}O_3$ , was expected to be a monohydroxydiacetate. Carbon and hydrogen analyses, however, strongly suggested that the product was a dihydroxymonoacetate rather than the expected monohydroxydiacetate.

On oxidation of this product with chromic acid anhydride in the cold, a substance was obtained in excellent yield which melted at 178°. After several crystallizations the melting point was raised to 191–192°. The carbon and hydrogen analyses were in excellent agreement with those required for a diketomono-

<sup>2</sup> The authors are deeply grateful to E. R. Squibb and Sons and to Dr. André Girard of Paris for gifts of urine extract, and to Dr. R. D. H. Heard of the Connaught Laboratories for gifts of extract and of isolated crystalline material. Without this generous help the continuance of this work would have been impossible.

acetate of the formula  $C_{21}H_{31}O_2(OCOCH_3)$ , but were in poor agreement with those required for a monoketodiacetate. On treatment of this compound with semicarbazide in the cold, a product was obtained which, judging from the observed nitrogen content, was unquestionably a disemicarbazone. It was thus certain that the hydrolysis of the triacetate with 1 equivalent of alkali had resulted in the removal of two acetyl groups instead of one. This unexpected result can obviously best be explained by the supposition that two of the three acetyl groups in the acetate have approximately the same degree of lability, so that on partial saponification they are simultaneously removed.

Since the "diketomonoacetate" failed to give any oxonium salt in acid solution, and since it formed no derivative with *o*-phenylenediamine, it seemed probable that it could not be an  $\alpha$ -diketone. Since it was not found to possess any demonstrable acidic properties, it seemed unlikely that it was a  $\beta$ -diketone. However, it was found that it reacted with hydrazine hydrate to form a pyridazine derivative which gave analytical results in close agreement with those required for the formula  $C_{23}H_{34}O_2N_2$ . This behavior is characteristic of both  $\beta$ - and  $\gamma$ -diketones. Since the lack of acidic properties is incompatible with the former possibility, it is clear that the "diketomonoacetate" must be a  $\gamma$ -diketone. If the assumption that the parent alcohol is a steroid arising from progesterone with hydroxyl groups at  $C_3$  and  $C_{20}$  is correct, then it follows that it is probably a pregnane- or allopregnane-3( $\alpha$ ),6,20-triol. It might be supposed that such a compound could be formed from progesterone by a shifting of the double bond from the 4-5 to the 5-6-position after reduction of the  $C_3$  ketone group, and subsequent addition of the elements of water, according to the following scheme.



The "diketomonoacetate" on reduction by the Wolff-Kishner method yielded a waxy crystalline product melting at 82–83°. The solubilities, melting point, and superficial appearance of



this substance suggested that it might be a hydrocarbon. On analysis, however, carbon and hydrogen figures closely agreeing with those required for a monohydroxy compound of the formula  $C_{21}H_{36}O$  were obtained. The presence of a hydroxyl group in this substance was confirmed by the preparation of a benzoate.

It has not been possible so far to determine the molecular weights of any of the derivatives of the "pregnanetriol" by the Rast method. The compounds described in this paper were either insoluble in molten camphor, or underwent decomposition below the melting point of the mixture. However, the preparation of the "dihydroxymonoacetate," the "diketomonoacetate," and the final product which contains one-third of the number of hydroxyl groups possessed by the original compound, conclusively proves that the latter must be represented by the formula  $(C_7H_{12}O)_3$ . It is therefore justifiable perhaps to assume that it is a  $C_{21}H_{36}O_3$  triol.

In view of the impossibility of reconciling the experimental results described in this paper with the conclusions arrived at by Marker *et al.* (4), it is necessary to discuss critically certain of the results obtained by these workers. They claim that the partial saponification of "pregnanetriol B triacetate," under conditions more vigorous than those employed by the present authors, followed by oxidation with chromic anhydride without isolation of the partial hydrolysis product, yielded a monoketodiacetate. The analytical figures reported support this conclusion, but it is perhaps suggestive that the melting point of the product (188°) is close to that of the diketomonoacetate (191–192°) described in the present paper. Marker *et al.* (4) have reported that this compound formed a semicarbazone, but since no analytical figures are reported, this fact has no particular significance. The present authors have repeated the partial hydrolysis of the triacetate and the oxidation of the resulting product many times, and they have on each occasion observed that the final product is undoubtedly a diketomonoacetate and not a monoketodiacetate.

Marker *et al.* (4) claimed that their "monoketodiacetate" yielded allopregnane by a Clemmensen reduction. They have explained this curious result by postulating that the ketone group in the "ketodiacetate" is at  $C_{20}$ , and that during the reaction dehydration of the 3,4-glycol formed by the hydrolytic removal

of the two acetyl groups gives rise to a  $C_3$  or  $C_4$  ketonic group, which is then reduced. The only evidence presented that the final product is allopregnane is that its melting point ( $80-81^\circ$ ) was not depressed by admixture with allopregnane (m.p.  $84^\circ$ ), but was depressed by admixture with pregnane (m.p.  $75^\circ$ ). Apparently no carbon and hydrogen analyses were carried out, since no analytical figures are reported.

If, as the present authors suspect, the "monoketodiacetate" prepared by these workers is really a diketomonoacetate, it seems that the Clemmensen reduction product may have been the mono-hydroxy compound  $C_{21}H_{36}O$  (m.p.  $82-83^\circ$ ) described by the present authors, and not allopregnane. The fact that it showed no depression of melting point on being mixed with allopregnane cannot alone be accepted as evidence of its identity with that hydrocarbon, since mixed melting points in the steroid group are notoriously unreliable. In this connection it may be recalled that Butler and Marrian (5) showed that the melting point of pregnane-3( $\alpha$ ),17,20-triol was not depressed by admixture with pregnane-3( $\alpha$ ),20-diol. It is conceivable that the compound  $C_{21}H_{36}O$  is allopregnane-20-ol, in which case the failure of its melting point to be depressed by admixture with allopregnane might be understandable.

#### EXPERIMENTAL

*Partial Hydrolysis of the Acetate ( $C_{21}H_{42}OCOCH_3$ )<sub>n</sub>*.—To 589 mg. of the acetate (m.p.  $167^\circ$ ) dissolved in the minimum volume of absolute methanol were added 57.12 mg. of potassium hydroxide dissolved in methanol. After standing for 30 hours at room temperature, the solution was made slightly acidic with acetic acid and evaporated to dryness under reduced pressure. The potassium acetate was removed from the mixture by leaching several times with hot water and the insoluble residue, after thorough drying, was repeatedly extracted with hot benzene. The benzene solution, on evaporation to dryness, yielded 254 mg. of a white solid which melted at  $222-224^\circ$ .<sup>3</sup>

5.311 mg. gave 14.145 mg.  $CO_2$  and 4.740 mg.  $H_2O$

$C_{21}H_{34}O(OCOCH_3)_2$ . Calculated. C 71.39, H 9.60

$C_{21}H_{36}O_2(OCOCH_3)_2$ . " " 72.96, " 10.13

Found. " 72.64, " 9.99

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<sup>3</sup> The melting points reported in this paper are uncorrected.

*Oxidation of the Hydroxyacetate*—To 247 mg. of the hydroxyacetate dissolved in 10 ml. of 90 per cent acetic acid were added 252 mg. of chromic anhydride in 10 ml. of 90 per cent acetic acid. After standing at room temperature for 12 hours, the mixture was warmed with 5 ml. of ethanol in order to destroy the excess of chromic acid, and then heavily diluted with water. The solid that separated out was filtered off, washed thoroughly with water, dried, and repeatedly crystallized from aqueous methanol. The final product was in the form of white needles, m.p. 191–192°.

3.446 mg. gave 9.305 mg.  $\text{CO}_2$  and 2.836 mg.  $\text{H}_2\text{O}$

$\text{C}_{21}\text{H}_{32}\text{O}(\text{OCOCH}_3)_2$ . Calculated. C 71.72, H 9.16

$\text{C}_{21}\text{H}_{31}\text{O}_2(\text{OCOCH}_3)$ . " " 73.74, " 9.16

Found. " 73.62, " 9.18

161 mg. of the ketoacetate were treated in ethanolic solution with an excess of semicarbazide hydrochloride and sodium acetate for 5 days at room temperature. The crude semicarbazone was separated from the mixture by evaporation of the ethanol and by leaching the residue thoroughly with water. This material could not be induced to crystallize, but on cooling a hot, saturated, filtered solution in acetone, it was obtained as a nearly white amorphous solid (yield, 115 mg.), which did not melt below 305°, but which underwent very slight decomposition at 220–223°.

2.859 mg. gave 0.416 ml.  $\text{N}_2$  at 20.5° and 762 mm.

$\text{C}_{25}\text{H}_{40}\text{O}_4\text{N}_6$ . Calculated, N 17.20; found, N 16.91

*Treatment of the Ketoacetate with Hydrazine Hydrate*—To 63.4 mg. of the ketoacetate dissolved in 5 ml. of ethanol was added 0.4 ml. of hydrazine hydrate (sp.gr. 1.032 at 20°), and the mixture was then heated at 80° for 5 hours. After evaporation of the mixture under reduced pressure, the residue was dried over concentrated sulfuric acid *in vacuo*, and then extracted thoroughly with hot benzene. By evaporation of the benzene to a small volume and addition of a large volume of hexane, the product was obtained as a light colored amorphous solid. Purification was effected by five successive precipitations from benzene solution with hexane. The final product was obtained in a yield of 14.6 mg. It decomposed slowly from 210° onwards, but did not melt below 310°.

2.379 mg. gave 6.470 mg.  $\text{CO}_2$  and 1.969 mg.  $\text{H}_2\text{O}$

2.162 " yielded  $\text{NH}_3$  equivalent to 1.11 ml. of 0.01001 N acid (Friedrich)  
 $\text{C}_{22}\text{H}_{14}\text{O}_2\text{N}_2$ . Calculated. C 74.54, H 9.26, N 7.57  
 Found. " 74.15, " 9.26, " 7.07

*Reduction of the Ketoacetate (Wolff-Kishner)*—102 mg. of the disemicarbazone of the ketoacetate were heated in a sealed tube for 12 hours at  $168\text{--}170^\circ$  with 0.3 gm. of Na dissolved in 5 ml. of ethanol. The reaction mixture was dissolved in water and the solution thoroughly extracted with ether. The ethereal extract, after being washed with water and evaporated to dryness, yielded 50 mg. of a gum which could not be induced to crystallize. This material was purified by repeated sublimations at  $80\text{--}100^\circ$  and 5 to 6 microns. After this treatment it was found possible to crystallize it from benzene-methanol. 11.2 mg. of long, white, blade-shaped crystals, m.p.  $82\text{--}83^\circ$ , were obtained.

3.008 mg. gave 9.104 mg.  $\text{CO}_2$  and 3.078 mg.  $\text{H}_2\text{O}$

$\text{C}_{21}\text{H}_{16}\text{O}$ . Calculated, C 82.82, H 11.93; found, C 82.54, H 11.45

7.2 mg. of this product were heated at  $80\text{--}90^\circ$  for 2 hours with 1 ml. of anhydrous pyridine and 0.5 ml. of benzoyl chloride. After dilution with water, the reaction mixture was thoroughly extracted with ether; the ether was washed successively with dilute sulfuric acid, sodium carbonate, and water, and evaporated to dryness. Benzoic acid was removed from the residue by sublimation at  $110^\circ$  for 3 hours at 6 microns. By sublimation at  $130^\circ$  a product consisting of crystals contaminated with an oil was obtained. Washing this with a small quantity of warm methanol removed the oil, leaving a small quantity of a white crystalline product which melted sharply at  $141^\circ$ .

1.025 mg. gave 3.103 mg.  $\text{CO}_2$  and 0.868 mg.  $\text{H}_2\text{O}$

$\text{C}_{11}\text{H}_{10}\text{OCOC}_6\text{H}_5$ . Calculated. C 82.28, H 9.87  
 Found. " 82.46, " 9.48

*Attempted Oxidation of the Substance  $(\text{C}_7\text{H}_{12}\text{O})_n$  with Lead Tetraacetate*—Two samples of the substance, weighing 9.1 and 9.0 mg. respectively, were each dissolved in 5 ml. volumes of an approximately 0.1 N solution of lead tetraacetate in glacial acetic acid. The two solutions together with a control were allowed to stand at room temperature for 24 hours. At the end of this

period 10 ml. of potassium iodide-sodium acetate solution (Criegee) were added to each flask and the liberated iodine titrated with 0.1 N sodium thiosulfate. The titrations for the two solutions containing the substance were 13.99 and 13.87 ml. respectively, while that of the control solution was 14.10 ml.

In another experiment 10.0 mg. of the substance were allowed to react with the lead tetraacetate solution for 24 hours at 37°. The thiosulfate titration was 13.87 ml. and that of the control 14.05 ml.

#### SUMMARY

The constitution of the saturated solid alcohol present in the urine of pregnant mares, which was first described by Smith *et al.* (1, 2), has been studied. Partial hydrolysis of the acetate yielded a product, m.p. 222–224°, which, on the assumption that the parent alcohol is a triol of the formula  $C_{21}H_{36}O_3$ , must be a dihydroxymonoacetate. On treatment of this "dihydroxymonoacetate" with chromic anhydride, a diketomonoacetate, m.p. 191–192°, was obtained. This diketomonoacetate was shown to be a  $\gamma$ -diketone by the preparation of a pyridazine derivative. By reduction of the diketomonoacetate by the Wolff-Kishner method, a monoatomic alcohol, m.p. 82–83°, was obtained.

It is tentatively suggested that the compound  $(C_7H_{12}O)_n$  is a pregnane- or allopregnane-3( $\alpha$ ),6,20-triol. The evidence put forward is incompatible with the conclusion of Marker *et al.* (4) that it is a pregnane-3( $\alpha$ ),4,20-triol.

The authors wish to express their gratitude to Miss Dorothy Skill and Dr. Helen Stantial for carrying out most of the microanalyses reported in this paper.

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## THE PREPARATION OF 3,4,5-TRIMETHYL L-GALACTONIC ACID

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In 1891 Thierfelder<sup>1</sup> discovered that it is possible to reduce the aldehydic group of uronic acids to the corresponding primary alcoholic group, without affecting the carboxyl group. By reduction with sodium amalgam he was able to transform *d*-glucuronic to *l*-gulonic acid (in a yield of 25 per cent) and Smolenski and Zlotnik<sup>2</sup> have recently prepared *l*-galactonic acid by treating *d*-galacturonic acid in the same manner.

The method has now been modified by performing the reduction with hydrogen under pressure, in the presence of Raney's catalyst, (the barium salt<sup>3</sup> of the uronic acid being used to prevent the acid dissolving some of the catalyst) and has been applied to the preparation of 3,4,5-trimethyl *l*-galactonic acid from 2,3,4-trimethyl *d*-galacturonic acid. The new trimethyl galactonic acid is unique among the known trimethyl galactonic acids in that it can give rise to neither a  $\gamma$ - nor a  $\delta$ -lactone. A comparable substance is the 3,4,5-trimethyl *d*-arabonic acid<sup>4</sup> recently obtained by treating pentamethylglucoascorbic acid with ozone.

The conditions employed for the above conversion may be applied to the preparation of a variety of partially substituted aldonic acids, certain of which are desired as reference compounds in investigations now in progress in this laboratory.

<sup>1</sup> Thierfelder, H., *Z. physiol. Chem.*, **15**, 71 (1891).

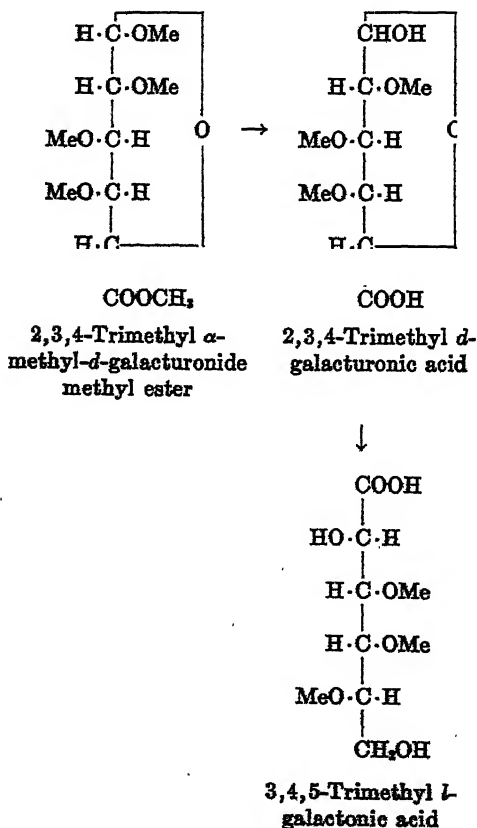
<sup>2</sup> Smolenski, K., and Zlotnik, A., *Bull. internat. acad. polonaise sc.*, 293 (1934).

<sup>3</sup> The device of using the barium salt was evolved during other work in this laboratory.

<sup>4</sup> Haworth, W. N., Hirst, E. L., and Jones, J. K. N., *J. Chem. Soc.*, 549 (1937).

The new crystalline 2,3,4-trimethyl *d*-galacturonic acid described in the present communication was prepared by the hydrolysis of the methyl ester of 2,3,4-trimethyl  $\alpha$ -methyl-*d*-galacturonide, the structure of which had already been proved by oxidation experiments.<sup>5</sup> The acid crystallizes in the  $\alpha$  form as the monohydrate and shows a fairly rapid mutarotation in water (presumably accelerated by its own carboxyl group), but lactone formation is impossible because of the substitution of the hydroxyl groups at positions (2) and (3).

For comparison, the velocity of hydrolysis of 2,3,4-trimethyl



<sup>5</sup> Levene, P. A., and Kreider, L. C., *J. Biol. Chem.*, **120**, 597 (1937).

$\alpha$ -methyl-*d*-galactoside<sup>6</sup> under the same conditions was also investigated and found to be of the same order.

The trimethyl galacturonic acid was now converted to the barium salt and this was hydrogenated in the presence of Raney's catalyst to give the barium salt of 3,4,5-trimethyl *l*-galactonic acid. Removal of the barium yielded the free acid, which was isolated as colorless crystals having  $[\alpha]_D^{27} = +12.6^\circ$  (in water), a specific rotation comparable in magnitude to, but opposite in sign from, that of *d*-galactonic acid.

#### EXPERIMENTAL

*Preparation of 2,3,4-Trimethyl d-Galacturonic Acid*—The course of hydrolysis at  $100^\circ$  of a 1 per cent solution of the crystalline methyl ester of 2,3,4-trimethyl  $\alpha$ -methyl-*d*-galacturonide<sup>5</sup> in *N* hydrochloric acid was studied polarimetrically, the changes in specific rotation being as follows:  $[\alpha]_D^{26} = +166.2^\circ$  (initial),  $+153.0^\circ$  (10 minutes),  $+142.7^\circ$  (20 minutes),  $+132.0^\circ$  (30 minutes),  $+118.3^\circ$  (60 minutes),  $+111.9^\circ$  (1.5 hours),  $+109.0^\circ$  (2 hours),  $+105.6^\circ$  (2.5 hours), and  $+104.1^\circ$  (3 hours), constant thereafter. Calculated as trimethyl hexuronic acid, the final specific rotation is  $[\alpha]_D^{26} = +116.5^\circ$  (in *N* hydrochloric acid).

The solution was cooled to room temperature, silver carbonate was added until all the hydrochloric acid had been neutralized, the mixture was filtered, and hydrogen sulfide was passed into the filtrate until all the silver had been precipitated. The mixture was now aerated, filtered, and the filtrate evaporated to dryness, giving a crystalline mass in quantitative yield. It was recrystallized from acetone, being obtained as colorless needles having a melting point of  $96-98^\circ$  and the following specific rotation in water.

$$[\alpha]_D^{27} = \frac{+2.91^\circ \times 100}{2 \times 1.152} = +126.3^\circ \text{ (4 minutes after dissolution)}$$

changing to  $+108.9^\circ$  (20 minutes), and  $+104.2^\circ$  (60 minutes), constant thereafter. Calculated as the anhydrous substance, the final specific rotation is  $[\alpha]_D^{27} = +112.1^\circ$  (in water).

<sup>6</sup> Levene, P. A., Tipson, R. S., and Kreider, L. C., *J. Biol. Chem.*, **122**, 199 (1937-38).



Its composition was as follows:

4.592 mg. substance: 7.205 mg. CO<sub>2</sub> and 2.995 mg. H<sub>2</sub>O  
 3.580 " " : 24.97 cc. 0.01 N sodium thiosulfate  
 C<sub>8</sub>H<sub>14</sub>O<sub>7</sub> · H<sub>2</sub>O. Calculated. C 42.50, H 7.1, OCH<sub>3</sub> 36.62  
 Found. " 42.78, " 7.3, " 36.05

*Rate of Hydrolysis of 2,3,4-Trimethyl α-Methyl-d-Galactoside—*

The course of hydrolysis at 100° of a 1 per cent solution of 2,3,4-trimethyl α-methyl-d-galactoside<sup>6</sup> in N hydrochloric acid (having an initial  $[\alpha]_D^{27} = +199.1^\circ$ ) was studied polarimetrically. The specific rotation showed a smooth change as follows: +130.4° (30 minutes), +114.4° (1 hour), +110.6° (2 hours), +109.7° (2.5 hours), constant thereafter. Calculated as trimethyl hexose, the final specific rotation is  $[\alpha]_D^{27} = +116.6^\circ$ .

*Catalytic Reduction of 2,3,4-Trimethyl d-Galacturonic Acid—*

1 gm. of crystalline 2,3,4-trimethyl d-galacturonic acid hydrate was dissolved in 50 cc. of water, a slight excess of barium carbonate was added, and the mixture was warmed at 60° until the solution was neutral to litmus. The mixture was then filtered and the filtrate diluted to 100 cc. with water.

An aqueous suspension of about 1 gm. of Raney's nickel catalyst was now added and the mixture shaken overnight in an atmosphere of hydrogen at a pressure of 3000 pounds per sq. inch at 125°.

The mixture was cooled, filtered with suction through a thin layer of charcoal, and the clear, colorless filtrate evaporated to dryness, giving 1.2 gm. of a colorless, flaky glass. This was dissolved in water and the barium removed quantitatively by adding N sulfuric acid dropwise.

The barium sulfate was removed by centrifuging and the clear, colorless solution evaporated to dryness, giving a solid mass (weight 0.9 gm.) of colorless crystals (rosettes of long needles). The substance was not very soluble in ether, chloroform, or benzene but was recrystallized from acetone. It had a melting point of 161–162° and the following specific rotation, with no mutarotation.

$$[\alpha]_D^{27} = \frac{+0.25^\circ \times 100}{2 \times 0.990} = +12.6^\circ \text{ (in water)}$$

Its composition was as follows:

4.823 mg. substance: 7.990 mg. CO<sub>2</sub> and 3.245 mg. H<sub>2</sub>O  
 3.316 " " : 24.94 cc. 0.01 N sodium thiosulfate  
 C<sub>8</sub>H<sub>14</sub>O<sub>7</sub>. Calculated. C 45.35, H 7.6, OCH<sub>3</sub> 39.08

## CONVERSION OF URONIC ACIDS INTO CORRESPONDING HEXOSES

### V. TRANSFORMATION OF THE ALDOBIONIC ACID (FROM GUM ARABIC) TO THE CORRESPONDING DISACCHARIDE

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As outlined in the preceding papers of this series,<sup>1-3</sup> we have engaged in a search for methods of converting uronic acids (and their more complex derivatives, including the aldobionic acids and pectins) into the corresponding hexose derivatives, by reduction of the carboxyl group.

In order to investigate suitable derivatives in which the reducing group of an aldobionic acid is protected and to ascertain the experimental conditions under which the ester group may be reduced without hydrogenolysis of the sugar chain or deoxygenation, we have chosen the aldobionic acid from gum arabic for preliminary study, since it is readily prepared, in relatively large quantity, in pure crystalline form.

When gum arabic is partially hydrolyzed with dilute mineral acid, it gives rise to an aldobionic acid which has been shown to be a glucuronidogalactose, since on complete hydrolysis it yields an equimolecular mixture of glucuronic acid and galactose.<sup>4,5</sup> The

<sup>1</sup> Levene, P. A., and Kreider, L. C., *J. Biol. Chem.*, **121**, 155 (1937).

<sup>2</sup> Levene, P. A., Tipson, R. S., and Kreider, L. C., *J. Biol. Chem.*, **122**, 199 (1937-38).

<sup>3</sup> Levene, P. A., and Christman, C. C., *J. Biol. Chem.*, **122**, 203, 661 (1937-38).

<sup>4</sup> Heidelberger, M., and Kendall, F. E., *J. Biol. Chem.*, **84**, 639 (1929).

<sup>5</sup> Heidelberger, M., Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, **49**, 847 (1929). Butler, C. L., and Cletcher, L. H., *J. Am. Chem. Soc.*, **51**, 1519 (1929).

precise structure of the aldobionic acid is, however, still unknown since the glucuronic acid residue might be an  $\alpha$ - or a  $\beta$ -glycoside.

If a reducing sugar is hydrogenated in the presence of Raney's nickel catalyst (2.5 hours at 150° and 2550 pounds per sq. inch pressure),<sup>6</sup> the aldehydic group is transformed to a primary alcoholic group; a carbomethoxy group remains unchanged under these conditions.

On the other hand, hydrogenation over copper chromite, under suitable conditions, causes reduction of both the carbonyl and the ester groups to primary alcoholic groups. As described by Adkins and coworkers,<sup>7,8</sup> the latter transformation requires more drastic conditions (3000 to 4500 pounds per sq. inch at 250°) but it has also been observed that<sup>9</sup> under this more vigorous treatment, a variety of sugars and their derivatives undergo hydrogenolysis with the formation of such substances as methanol, ethanol, and propane-1,2-diol.

In order to effect the transformation of an aldobionic acid to the corresponding disaccharide it is therefore necessary to protect the reducing aldehydic group from attack and also to choose conditions of hydrogenation sufficiently drastic to reduce the ester without causing appreciable hydrogenolysis or deoxygenation.

Following upon our recent announcement<sup>2,10</sup> of a method for the conversion of uronic acids to the corresponding hexoses (by catalytic hydrogenation) we have sought to apply the method to aldobionic acids, in such a manner that the corresponding disaccharide should be isolated *as such* (not as some methylated, or otherwise substituted, derivative).

This has now been accomplished in two different ways. One or the other of these types of reaction should prove of applicability to most aldobionic acids.

#### *Reduction and Deacetylation of Hexaacetyl Methylaldobionide*

<sup>6</sup> Covert, L. W., Connor, R., and Adkins, H., *J. Am. Chem. Soc.*, **54**, 1651 (1932).

<sup>7</sup> Folkers, K., and Adkins, H., *J. Am. Chem. Soc.*, **54**, 1145 (1932). Adkins, H., and Folkers, K., *J. Am. Chem. Soc.*, **53**, 1095 (1931).

<sup>8</sup> Adkins, H., and Connor, R., *J. Am. Chem. Soc.*, **53**, 1091 (1931).

<sup>9</sup> Zartman, W. H., and Adkins, H., *J. Am. Chem. Soc.*, **55**, 4559 (1933).

<sup>10</sup> Levene, P. A., Tipson, R. S., and Kreider, L. C., *Science*, **86**, 332 (1937).

*Methyl Ester*—In another communication<sup>11</sup> we described a methylglycoside of 6-glucosidogalactose, formed by the catalytic reduction of a crystalline hexaacetyl methylaldobionide methyl ester from the aldobionic acid of gum arabic.

On the basis of its specific rotation this methylbioside appeared to be a  $\beta$ -methylglycoside, but there was no indication as to whether it were a pyranoside or a furanoside. We now find that the methoxyl group in glycosidic union has the instability towards very dilute mineral acid characteristic of furanosides—it is completely hydrolyzed to the free disaccharide on treatment with 0.01 N hydrochloric acid during 4 hours at 100°. Definite proof of its furanoside ring structure must await the outcome of methylation experiments, but the important point is that the product provides a path for the ready isolation of the free disaccharide (which is not hydrolyzed under the above conditions).

*Reduction and Deacetylation of the Triacetate of Diacetone 6-Glucuronidogalactose Methyl Ester*—The other method of converting the aldobionic acid (into the corresponding biose) consisted in the transformation of its crystalline methyl ester into the corresponding diacetone derivative (by condensation with acetone in the usual manner), the triacetate of which was identical with that synthesized from galactose and glucuronic acid by Hotchkiss and Goebel.<sup>12</sup>

Simultaneous reduction and deacetylation of this triacetate gave diacetone 6-glucosidogalactose which was readily hydrolyzed by dilute mineral acid to the corresponding biose.

Challinor and coworkers<sup>13</sup> methylated the aldobionic acid from gum arabic, obtaining the methyl ester of hexamethyl glucuronidomethylgalactoside which was hydrolyzed to 2,3,4-trimethyl glucuronic acid and a trimethylgalactose. This trimethylgalactose consisted largely of 2,3,4-trimethylgalactose, since on further methylation it gave a good yield of 2,3,4,6-tetramethyl  $\beta$ -methylgalactoside and, on oxidation of the trimethyl sugar with bromine, a trimethyl  $\delta$ -galactonolactone was obtained.

However, the properties recorded by Challinor *et al.*<sup>13</sup> for the

<sup>11</sup> Levene, P. A., and Tipson, R. S., *Science*, **86**, 593 (1937).

<sup>12</sup> Hotchkiss, R. D., and Goebel, W. F., *J. Biol. Chem.*, **115**, 285 (1936).

<sup>13</sup> Challinor, S. W., Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 258 (1931).

trimethylgalactose are not in agreement with those of pure 2,3,4-trimethylgalactose, as shown in the accompanying tabulation.

Challinor, Haworth, and Hirst<sup>13</sup>

Pale yellow, viscid syrup

$n_D^{20} = 1.4727$

$[\alpha]_D^{20} = +83^\circ$  (in  $H_2O$ )

Found,  $OCH_3 = 38.5$

Calculated, " = 41.9

Levene, Tipson, and Kreider<sup>14,15</sup>

Colorless crystals, m.p. 82-83°

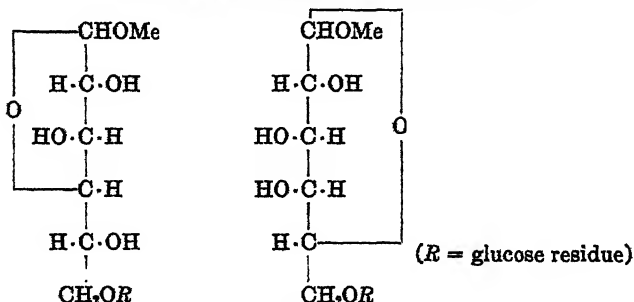
$n_D^{20} = 1.4810$  (superfused substance)

$[\alpha]_D^{20} = +156.0^\circ \rightarrow +119.1^\circ$  (equilibrium in  $H_2O$ )

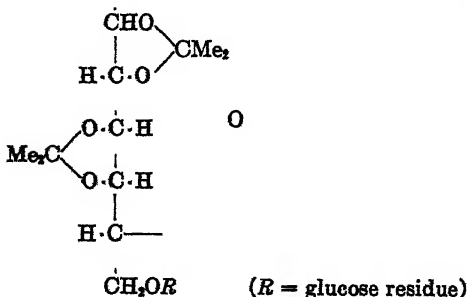
Found,  $OCH_3 = 41.97$

Calculated, " = 41.90

*Methylbioside (Substituted Methylgalactoside)*



*Diacetone Biose (Substituted Diacetone Galactose)*

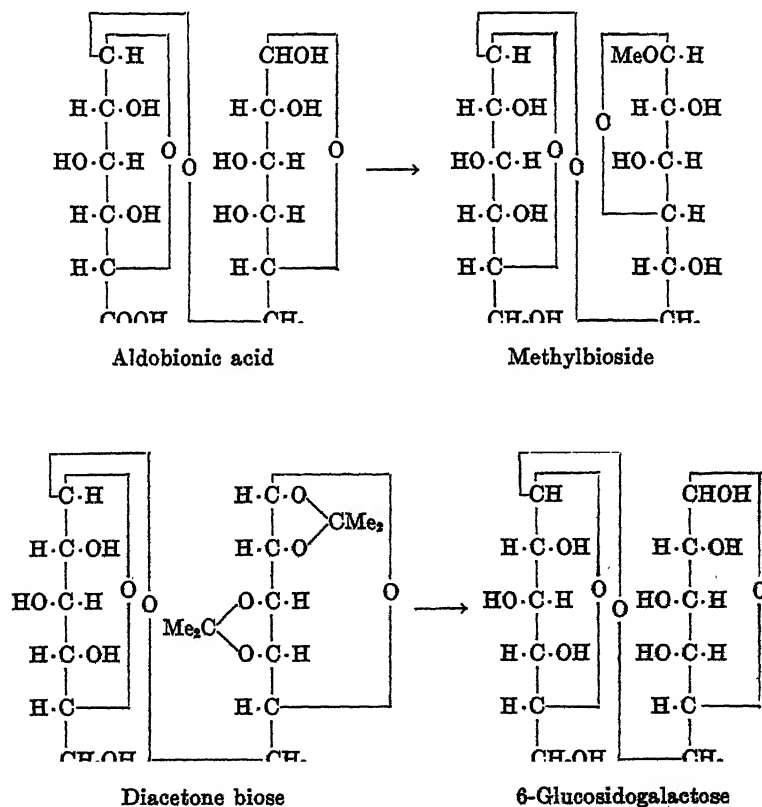


On the basis of these results, they assigned to the aldobionic acid the structure of a 6-glucuronopyranosidogalactose but were unable to reach a definite conclusion as to the configuration of the biose linkage. It should be noted that the galactose moiety can

<sup>14</sup> Compare also Onuki, M., *J. Agric. Chem. Soc. Japan*, 9, 90, 214 (1933); *Proc. Imp. Acad.*, Tokyo, 8, 496 (1933).

give rise to furanose or pyranose derivatives according to the conditions, whereas the glucuronic residue is fixed as a pyranoside.

This aldobionic acid was then synthesized by Hotchkiss and Goebel<sup>12</sup> by condensing diacetone galactose with the methyl ester of bromotriacetyl glucuronic acid, followed by deacetylation and hydrolysis of the acetone groups. This method of preparation of the aldobionic acid makes probable the  $\beta$  configuration of the glycosidic union for this substance.



#### EXPERIMENTAL

*Preparation of Hexaacetyl "β"-Methylaldobionide Methyl Ester*—3.25 gm. of "β"-methylaldobionide methyl ester<sup>4</sup> were dissolved in 25 cc. of pyridine. 20 cc. of acetic anhydride were added and

the mixture cooled in ice. After shaking for a few minutes, the solution was kept in the refrigerator overnight.

The product was isolated as described for tetraacetyl ribose,<sup>15</sup> giving a practically quantitative yield of glassy substance. After recrystallization from absolute methanol, it was obtained as colorless crystals (yield, 60 per cent), which had a melting point of 140° and the following composition.

5.029 mg. substance:	9.081 mg. CO <sub>2</sub> and 2.593 mg. H <sub>2</sub> O
4.425 " "	: 8.59 cc. 0.01 N sodium thiosulfate (methoxyl)
5.805 " "	: 5.53 " 0.01 " " (acetyl)
C <sub>22</sub> H <sub>38</sub> O <sub>18</sub> . Calculated.	C 49.04, H 5.7, OCH <sub>3</sub> 9.75, COCH <sub>3</sub> 40.57
Found.	" 49.24, " 5.8, " 10.01, " 40.96

Its specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{-1.20^\circ \times 100}{2 \times 1.106} = -54.2^\circ \quad (\text{in acetone})$$

*Simultaneous Catalytic Reduction and Deacetylation of Hexaacetyl "β"-Methylaldobionide Methyl Ester*—2.5 gm. of dry recrystallized methyl ester of hexaacetyl "β"-methylaldobionide were dissolved in 100 cc. of absolute methanol. 2.5 gm. of copper chromite catalyst<sup>8</sup> were added and the mixture was shaken with hydrogen at a pressure of 3600 pounds per sq. inch during 5 hours at 175°.

The mixture was now cooled and the black catalyst removed by filtration. Evaporation of the clear, colorless filtrate gave a colorless glass, weight 1.4 gm. The product was dissolved in a little water and extracted several times with chloroform but evaporation of the chloroform solution gave no trace of material, indicating complete deacetylation.

The aqueous solution was reevaporated to dryness and freed from traces of water by repeated evaporation with absolute ethanol and benzene, giving a colorless glass which was non-reducing to boiling Fehling's solution and was quite free from uronic acid (naphthoresorcinol test). It had a composition agreeing approximately with that of a methylbioside.

<sup>15</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **92**, 109 (1931).

4.686 mg. substance: 7.605 mg. CO<sub>2</sub> and 2.905 mg. H<sub>2</sub>O

4.215 " " : 8.02 cc. 0.01 N thiosulfate

C<sub>13</sub>H<sub>24</sub>O<sub>11</sub>. Calculated. C 43.80, H 6.8, OCH<sub>3</sub> 8.71

Found. " 44.25, " 6.9, " 9.84

These analytical results suggested the presence of a trace of material containing less oxygen, since the composition of a monodesoxy methylbioside would be C<sub>13</sub>H<sub>24</sub>O<sub>10</sub>, calculated, C 45.86, H 7.1, OCH<sub>3</sub> 13.80.

In subsequent experiments, therefore, the reduction was only permitted to proceed during 3 hours at 175°. The product was isolated as described above and its aqueous solution extracted with chloroform, a trace of unchanged acetate being recovered in the chloroform extract.

The aqueous layer was evaporated to dryness and the flaky glass-like product, obtained in almost quantitative yield, had the following composition.

3.529 mg. substance: 5.675 mg. CO<sub>2</sub> and 2.330 mg. H<sub>2</sub>O

C<sub>13</sub>H<sub>24</sub>O<sub>11</sub>. Calculated, C 43.80, H 6.8; found, C 43.85, H 7.3

Its specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{-1.63^\circ \times 100}{2 \times 1.172} = -69.5^\circ \text{ (in water)}$$

*Hydrolysis of Methylbioside to Free Biose*—The course of hydrolysis (of the methoxyl group) at 100° of a 1 per cent solution of the methylbioside in 0.01 N aqueous hydrochloric acid solution was studied polarimetrically. Under these conditions, the initial specific rotation of the solution ( $[\alpha]_D^{25} = -77.6^\circ$ ) showed a smooth change as follows:  $-54.3^\circ$  (30 minutes),  $-38.6^\circ$  (1 hour),  $-16.7^\circ$  (2.25 hours),  $+4.8^\circ$  (3 hours),  $+12.4^\circ$  (4, 5, and 6 hours). Recalculated as free biose, the final specific rotation is  $[\alpha]_D^{25} = +13.0^\circ$ .

Under the same conditions, the synthetic crystalline 6-glucosidogalactose likewise showed no observable scission of the biose linkage. (The equilibrium rotation of a solution containing a mixture of equal weights of *d*-galactose and *d*-glucose is calculated to be  $[\alpha]_D^{25} = +66.5^\circ$ .)

1.5 gm. of the methylbioside were therefore dissolved in 150 cc. of 0.01 N aqueous sulfuric acid and the solution heated on the steam bath under a reflux condenser during 4 hours. The solution was



cooled in ice to 25°, the free mineral acid neutralized with barium carbonate, and the mixture filtered. The clear colorless filtrate was evaporated to dryness under diminished pressure to a colorless glass (weight 1.4 gm.). This was dissolved in 15 cc. of absolute methanol, 15 cc. of absolute ethanol were added, and a trace of insoluble impurity was removed by filtration. On evaporating slowly in a desiccator at room temperature, the solution deposited colorless crystals of the disaccharide. The substance, which was strongly reducing to boiling Fehling's solution, had a melting point of 126–128° and the following composition.

5.398 mg. substance:	7.995 mg. CO <sub>2</sub> ,	3.120 mg. H <sub>2</sub> O,	and 0.265 mg. ash
	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> .	Calculated.	C 42.08, H 6.5
		Found (ash-free).	" 42.47, " 6.8

Its specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{+0.29^\circ \times 100}{2 \times 1.023} = +14.2^\circ \quad (\text{equilibrium in water})$$

*Preparation of Methyl Ester of 2,3,4-Triacetyl 6-β-Glucuronidogalactose Diacetone from Natural Aldobionic Acid*—The free aldobionic acid did not condense with acetone on shaking at room temperature in the presence of sulfuric acid as catalyst. The methyl ester of the aldobionic acid, however, condensed quite rapidly under these conditions, without hydrolysis of the methyl ester group. In preliminary experiments the resulting diacetone methyl aldobionate was isolated in the usual manner, giving a white powder which was very soluble in absolute methanol but very sparingly soluble in dry ether. Since the product was difficult to purify, it was acetylated and isolated as the crystalline triacetate.

2 gm. of dry, finely powdered crystalline methyl aldobionate<sup>12</sup> (recrystallized from twice its weight of absolute methanol) were shaken overnight at room temperature (25°) with a solution of 0.2 cc. of concentrated sulfuric acid in 200 cc. of acetone, after which time the ester had dissolved completely to a clear, colorless solution. 25 cc. of dry pyridine and 20 cc. of acetic anhydride were now added and the solution evaporated under diminished pressure to a volume of 45 cc. and allowed to stand overnight at room temperature.

The solution was poured into 1 liter of filtered ice water, the

white powdery precipitate filtered off, washed with water until free from pyridine, and dried in a vacuum desiccator. Weight, 1 gm. A further quantity (1.5 gm.) was isolated from the aqueous solution by extraction with chloroform. The chloroform extract was washed successively with ice-cold dilute sulfuric acid, water, sodium bicarbonate, water, and then dried over anhydrous sodium sulfate. The mixture was filtered and the filtrate evaporated to dryness under diminished pressure. It was dissolved in dry ether, pentane was added to faint opalescence, and on being nucleated with an authentic specimen of the synthetic methyl ester of triacetyl 6- $\beta$ -glucuronidogalactose diacetone<sup>16</sup> the substance crystallized to a solid mass of long needles.

It was recrystallized from 50 per cent ethyl alcohol (1.5 gm. in 10 cc. of solvent) giving colorless, fine needles.

The crystalline product had a melting point of 112° and the following specific rotation.

$$[\alpha]_D^{25} = \frac{-1.80^\circ \times 100}{2 \times 1.358} = -66.3^\circ \text{ (in chloroform)}$$

Its composition was as follows:

4.919 mg. substance:	9.445 mg. CO <sub>2</sub> and 2.810 mg. H <sub>2</sub> O
4.680 " "	5.30 cc. 0.01 N sodium thiosulfate (methoxyl)
8.594 " "	4.51 " 0.01 " " (acetyl)
C <sub>25</sub> H <sub>38</sub> O <sub>15</sub> .	Calculated. C 52.06, H 6.3, OCH <sub>3</sub> 5.38, COCH <sub>3</sub> 22.40
	Found. " 52.36, " 6.4, " 5.85, " 22.56

*Catalytic Reduction of Methyl Ester of Triacetyl Diacetone Aldobionic Acid to Diacetone Biose*—2.0 gm. of dry, recrystallized methyl ester of triacetyl diacetone aldobionic acid were dissolved in 100 cc. of absolute methanol, 2.0 gm. of copper chromite catalyst were added, and the mixture was shaken in an atmosphere of hydrogen at a pressure of 4000 pounds per sq. inch during 5 hours at 175°.

The mixture was now cooled, filtered with suction through a thin layer of charcoal, and the catalyst (which was still black) washed thoroughly with acetone. The clear, colorless filtrate and washings were combined and evaporated to dryness, giving a

<sup>16</sup> Kindly supplied by Dr. Walther F. Goebel.

colorless glass (weight 1.45 gm.). The product was quite free from uronic acid (naphthoresorcinol test), non-reducing to boiling Fehling's solution, and had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-1.41^\circ \times 100}{2 \times 1.026} = -68.7^\circ \quad (\text{in water})$$

Freudenberg *et al.*<sup>17</sup> report  $[\alpha]_D^{20} = -67.5^\circ$  (in water) for synthetic diacetone 6- $\beta$ -glucosidogalactose.

*Hydrolysis of Diacetone Biose to Free Biose*—Polarimetric observation showed that the hydrolysis of the isopropylidene groups from the diacetone biose (a 1 per cent solution) was complete after treatment during 30 minutes at 100° with 0.01 N aqueous hydrochloric acid, the initial specific rotation,  $[\alpha]_D^{26} = -67.7^\circ$ , changing to the constant value,  $[\alpha]_D^{26} = +11.7^\circ$ . Recalculated as free biose, the final specific rotation is  $[\alpha]_D^{26} = +14.5^\circ$ .

1 gm. of the diacetone biose was now dissolved in 100 cc. of 0.01 N aqueous sulfuric acid and the solution heated during 30 minutes at 100°. The solution was cooled in ice to 25°, and the product isolated as described for the hydrolysis of the methylbioside. Slow evaporation of a solution of the biose in methanol-ethanol gave a crystalline product, which was strongly reducing to boiling Fehling's solution, having a melting point of 128°.

The substance had the following composition.

5.111 mg. substance: 7.870 mg. CO<sub>2</sub> and 3.030 mg. H<sub>2</sub>O

C<sub>13</sub>H<sub>22</sub>O<sub>11</sub>. Calculated, C 42.08, H 6.5; found, C 42.00, H 6.6

Its specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{+0.30^\circ \times 100}{2 \times 1.072} = +14.0^\circ \quad (\text{equilibrium in water})$$

<sup>17</sup> Freudenberg, K., Noë, A., and Knopf, E., *Ber. chem. Ges.*, **60**, 238 (1937).

## CONVERSION OF URONIC ACIDS INTO CORRESPONDING HEXOSES

### VI. CONFIGURATION OF THE GLYCOSIDIC UNION OF THE ALDOBIONIC ACID FROM GUM ARABIC

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New York)

(Received for publication, June 22, 1938)

It was stated in the preceding paper<sup>1</sup> that the conversion of aldobionic acids to bioses may serve to bring out certain details of their structure which cannot be conveniently revealed without such conversion. A striking illustration is the question of the configuration of the glycosidic union between the two components of the aldobionic acids.

Helfferich<sup>2</sup> has shown on many occasions that substitution on carbon atom (6) of glycosides inhibits the action of hydrolytic enzymes on the groups on carbon atom (1). It was natural to expect that glycosides of uronic acids likewise would be resistant towards the action of such enzymes. This actually is true in regard to the action of emulsin on the aldobionic acid of gum arabic. On the other hand, the *6-glucosidodulcitol* derived from the aldobionic acid is readily hydrolyzed by emulsin and by this reaction the configuration of the glycosidic union of the aldobionic acid is established as that of *6-β-glucuronidogalactose*.

Inasmuch as *6-glucosidogalactose* has been prepared synthetically by Freudenberg *et al.*,<sup>3</sup> it was considered desirable to compare the properties of this substance and its derivatives with the properties of the biose derivatives obtained by reduction of the

<sup>1</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **125**, 345 (1938).

<sup>2</sup> Helfferich, B., in Nord, F. F., and Weidenhagen, R., *Ergebnisse der Enzymforschung*, Leipsic, **2**, 74 (1933).

<sup>3</sup> Freudenberg, K., Noë, A., and Knopf, E., *Ber. chem. Ges.*, **60**, 238 (1927).

aldobionic acid. The properties of the two sets of substances were found very similar.

Incidentally, occasion was taken to prepare the methyl ethers of the synthetic biose (of Freudenberg) to serve for comparison with the methylated biose obtained on reduction of the fully methylated aldobionic acid to be described later.

The conversion of the aldobionic acid to glucosidodulcitol was conducted in stages in the following way. Catalytic hydrogenation of the aldobionic acid in the presence of Raney's catalyst gave a *glucuronidodulcitol*, and the octaacetyl methyl ester of this product was simultaneously reduced and deacetylated in the presence of copper chromite catalyst to give a glucosidodulcitol which proved to be hydrolyzed by emulsin with the liberation of one free aldehydic group.

Catalytic reduction and deacetylation of the crystalline hepta-acetyl methyl ester of the aldobionic acid, in presence of copper chromite catalyst, gave crude glucosidodulcitol in one step but the product was impure and difficult to purify.

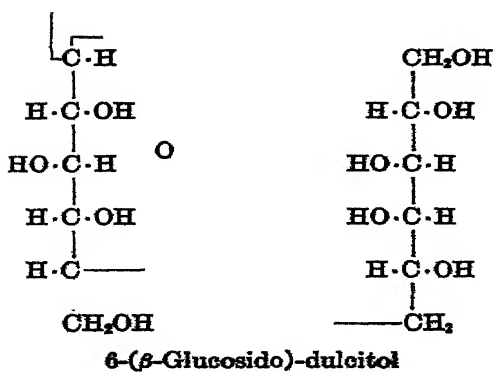
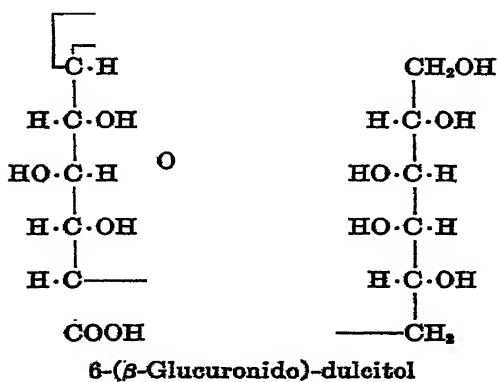
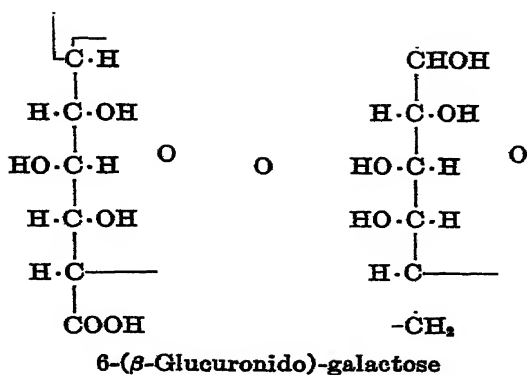
#### EXPERIMENTAL

*Conversion of Aldobionic Acid (from Gum Arabic) to 6-Glucuronidodulcitol*—5 gm. of dry, crystalline aldobionic acid dihydrate<sup>4</sup> (from gum arabic) were dissolved in 100 cc. of water. An aqueous suspension of about 5 gm. of Raney's nickel catalyst was added, and the mixture was shaken overnight in an atmosphere of hydrogen, at a pressure of 3000 pounds per sq. inch at 125°.

The mixture was now cooled and filtered with suction through a thin layer of charcoal. The filtrate was perfectly clear but had a pale green color owing to the presence of dissolved nickel. It was neutral to Congo red but acid to blue litmus, was non-reducing to boiling Fehling's solution (but strongly reducing after hydrolysis by mineral acid), and gave a strong test for uronic acid with naphthoresorcinol.

Excess finely powdered calcium carbonate was added (giving a solution neutral to blue litmus). The suspension was boiled and hydrogen sulfide was passed into the boiling suspension until no more black precipitate formed.

<sup>4</sup> Heidelberg, M., and Kendall, F. E., *J. Biol. Chem.*, **84**, 639 (1929).



The hot suspension was filtered with suction through a thin layer of charcoal and the clear, pale yellow filtrate cooled in ice to room temperature. A cold saturated aqueous solution of oxalic acid was now added until a small centrifuged test portion gave no precipitate on adding a drop of oxalic solution and warming.

The mixture was filtered and the colorless filtrate evaporated to dryness, giving an almost quantitative yield of a thick, colorless glass-like substance.

By slow evaporation of a concentrated aqueous solution in a vacuum desiccator it crystallized in rosettes of fine needles having a melting point of 179–182°, but from aqueous alcohol, colorless long rectangular platelets having a melting point of 132–135° were obtained.

After drying over phosphorus pentoxide at 100° and 12 mm. the substance had the following composition.

5.363 mg. substance: 7.887 mg. CO<sub>2</sub> and 3.250 mg. H<sub>2</sub>O

C<sub>12</sub>H<sub>12</sub>O<sub>12</sub>. Calculated, C 40.20, H 6.2; found, C 40.10, H 6.7

The substance had the following specific rotation, with no mutarotation.

$$[\alpha]_D^{25} = \frac{-0.50^\circ \times 100}{2 \times 1.152} = -21.7^\circ \quad (\text{in water})$$

*Preparation of Methyl Ester of 6-Glucuronidodulcitol*—3.5 gm. of dry 6-glucuronidodulcitol were dissolved in 300 cc. of boiling absolute methanol and the solution cooled to 0°. A cold, ethereal solution of diazomethane was slowly added, with shaking, until a faint yellow color persisted. The solution was then evaporated to dryness, giving a quantitative yield of a white powdery material which was stirred with 10 cc. of cold absolute methanol and the suspension filtered. After drying at 80° and 12 mm. over phosphorus pentoxide, the substance (which was non-reducing to boiling Fehling's solution, but contained uronic acid and was strongly reducing after acid hydrolysis) had a melting point of 83–85° (not sharp) and the following composition.

5.009 mg. substance: 7.690 mg. CO<sub>2</sub> and 3.000 mg. H<sub>2</sub>O

5.275 " " : 8.55 cc. 0.01 N sodium thiosulfate

C<sub>13</sub>H<sub>24</sub>O<sub>12</sub>. Calculated. C 41.91, H 6.5, OCH<sub>3</sub> 8.34

Found. " 41.86, " 6.7, " 8.38

Its specific rotation (with no mutarotation) was as follows:

$$[\alpha]_D^{25} = \frac{-0.57^\circ \times 100}{2 \times 1.046} = -27.3^\circ \quad (\text{in water})$$

*Preparation of Octaacetyl 6-Glucuronidodulcitol Methyl Ester*—To 1.5 gm. of the dry, finely powdered methyl ester of glucuronidodulcitol was added a cooled solution of 20 cc. of acetic anhydride in 25 cc. of dry pyridine at 0°. The mixture was shaken until the ester had dissolved. The solution was kept overnight at room temperature and then poured into 1 liter of filtered ice water with vigorous stirring.

The white powdery precipitate was filtered off, washed several times with distilled water, and dried (weight, 1.5 gm.). The aqueous filtrate was extracted with chloroform and, after pyridine and acetic acid were removed in the usual manner, the chloroform extract gave on evaporation a further 1.1 gm. of product as a colorless mass of fine hairy needles. It was recrystallized as follows: 1.5 gm. were dissolved in 15 cc. of boiling absolute methanol under a reflux condenser. The hot solution was filtered and cooled, whereupon a semigelatinous mass of fine hairy needles separated. The material was filtered off and dried, giving a white powder (weight 1 gm.), which was apparently no longer crystalline, but had a melting point of 154–155° and the following composition.

4.993 mg. substance:	9.000 mg. CO <sub>2</sub> and 2.622 mg. H <sub>2</sub> O
6.515 " "	: 5.78 cc. 0.01 N sodium thiosulfate (methoxyl)
7.224 " "	: 8.12 " 0.01 " " (acetyl)
C <sub>29</sub> H <sub>40</sub> O <sub>10</sub> .	Calculated. C 49.13, H 5.7, OCH <sub>3</sub> 4.38, COCH <sub>3</sub> 48.59
	Found. " 49.15, " 5.9, " 4.43, " 48.33

The substance had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.66^\circ \times 100}{2 \times 1.040} = -31.7^\circ \quad (\text{in acetone})$$

*Simultaneous Catalytic Reduction and Deacetylation of Octaacetyl 6-Glucuronidodulcitol Methyl Ester to 6-Glucosidodulcitol*—2.5 gm. of the methyl ester of octaacetyl glucuronidodulcitol were dissolved in 100 cc. of absolute methanol. 2.5 gm. of copper chromite



catalyst<sup>5</sup> were added and the mixture was shaken in an atmosphere of hydrogen at a pressure of 4500 pounds per sq. inch during 5 hours at 175°.

The product was then isolated in the usual manner, giving 1.1 gm. of a colorless, flaky glass which was quite free from uronic acid (naphthoresorcinol test), and was non-reducing to boiling Fehling's solution but strongly reducing after acid hydrolysis. It was isolated as a white powder by dissolving in methanol and adding dry ether. The product had the following composition.

4.085 mg. substance: 6.200 mg. CO<sub>2</sub> and 2.595 mg. H<sub>2</sub>O

C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>. Calculated, C 41.84, H 7.0; found, C 41.40, H 7.1

Its specific rotation, with no mutarotation, was as follows:

$$[\alpha]_D^{25} = \frac{-0.56^\circ \times 100}{2 \times 1.220} = -22.9^\circ \quad (\text{in water})$$

*Preparation of Tetraacetyl Glucosidogalactose Diacetone*—The method of Freudenberg *et al.*<sup>6</sup> for the condensation of diacetone galactose with bromoacetyl glucose was modified, giving a larger yield of the disaccharide derivative.

50 gm. of redistilled diacetone *d*-galactose were dissolved in 500 cc. of dry benzene. A mixture of 50 gm. of anhydrous calcium sulfate (drierite, No. 20 mesh) and 50 gm. of dry silver oxide were added and the mixture shaken for a few minutes.

80 gm. of dry, recrystallized bromotetraacetyl *d*-glucose were then added and the mixture shaken mechanically at room temperature overnight. A filtered test portion of solution now contained no bromine, so the product was isolated as follows:

The mixture was filtered, the solid material washed with acetone, and the combined filtrate and washings evaporated to dryness. The pale yellow gum was dissolved in 250 cc. of warm absolute ethanol, 250 cc. of water were added, and the solution was kept in the refrigerator overnight. The crystalline material was filtered off and dried (weight 57 gm.). A further 13 gm. of crystals were obtained from the filtrate.

It was recrystallized by dissolving 10 gm. in 25 cc. of boiling absolute ethanol. The solution was filtered while hot and on

<sup>5</sup> Adkins, H., and Connor, R., *J. Am. Chem. Soc.*, **53**, 1091 (1931).

cooling to room temperature deposited a first crop of 8.5 gm. of pure tetraacetyl diacetone glucosidogalactose having a melting point of 140°.

This substance was deacetylated and the acetone residues removed as described by Freudenberg, but since his method for recrystallizing the disaccharide proved unsatisfactory, the glucosidogalactose was recrystallized as follows:

15.5 gm. of the colorless, glass-like disaccharide were dissolved in 200 cc. of boiling absolute methanol under a reflux condenser. The hot solution was filtered, 10 cc. of absolute ethanol were added to the filtrate; and the solution was evaporated slowly in a vacuum desiccator at room temperature. 6-Glucosidogalactose separated in rosettes of long, rectangular, hard crystals having a melting point of 128–130° and (dried at 100°) the following composition.

4.880 mg. substance: 7.520 mg. CO<sub>2</sub> and 2.844 mg. H<sub>2</sub>O

C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>. Calculated, C 42.08, H 6.5; found, C 42.02, H 6.5

The  $\alpha$  form of the disaccharide was isolated, since its specific rotation in water was as follows:

$$[\alpha]_D^{25} = \frac{+1.09^\circ \times 100}{2 \times 1.594} = +34.2^\circ \quad (10 \text{ minutes after admixture})$$

$$[\alpha]_D^{25} = \frac{+0.47^\circ \times 100}{2 \times 1.594} = +14.7^\circ \quad (\text{equilibrium})$$

Freudenberg *et al.*<sup>6</sup> isolated the  $\beta$  form of this disaccharide having  $[\alpha]_D^{18} = +1.6^\circ$ , changing to  $+13.9^\circ (\pm 0.5^\circ)$  at equilibrium in water.

*Catalytic Reduction of Synthetic 6- $\beta$ -Glucosido- $\alpha$ -Galactose*—6 gm. of synthetic 6-glucosidogalactose were dissolved in 100 cc. of distilled water, an aqueous suspension of several gm. of Raney's catalyst was added, and the mixture was shaken overnight in an atmosphere of hydrogen at a pressure of 3000 pounds per sq. inch at 125°.

The mixture was now cooled and filtered with suction through a thin layer of charcoal. The clear, colorless filtrate was evapo-

<sup>6</sup> Freudenberg, K., Wolf, A., Knopf, E., and Zaheer, S. H., *Ber. chem. Ges.*, **61**, 1743 (1928).

rated to dryness, giving a colorless flaky glass (5.5 gm.) which dissolved completely in 50 cc. of boiling absolute methanol. The solution was cooled to room temperature, absolute ethanol was added to faint opalescence, and after standing overnight at room temperature, the product separated in colorless, long rectangular crystals. The first crop was filtered off, washed with a little cold methanol, and dried (weight 2.5 gm.). Further crops amounting to 2.5 gm. were obtained from the mother liquor. The crystalline material was not very soluble in absolute methanol, so it was recrystallized as follows:

2.5 gm. of crystalline substance were suspended in 100 cc. of boiling absolute methanol and a total volume of 7 cc. of water was added dropwise (until the material just dissolved in the boiling solvent). The hot solution was filtered and the filtrate cooled to room temperature. On nucleating and standing in the refrigerator, 2.2 gm. of crystalline product (m.p. 129–130°) separated. It was non-reducing to boiling Fehling's solution.

After drying over sulfuric acid at 100° and 12 mm., it had the following composition.

5.132 mg. substance: 7.905 mg. CO<sub>2</sub> and 3.220 mg. H<sub>2</sub>O

C<sub>13</sub>H<sub>24</sub>O<sub>11</sub>. Calculated, C 41.84, H 7.0; found, C 42.00, H 7.0

The air-dried crystalline substance, containing 3.93 per cent of water, had the following specific rotation, with no mutarotation.

$$[\alpha]_D^{24} = \frac{-0.40^\circ \times 100}{2 \times 1.024} = -19.5^\circ \text{ (in water)}$$

Calculated as the anhydrous substance this specific rotation is  $[\alpha]_D^{24} = -20.4^\circ$  (in water).

*Action of Emulsin on Synthetic Biose and Its Reduction Product. Synthetic 6-Glucosido- $\alpha$ -Galactose*—An emulsin solution was prepared as follows: 0.6 gm. of dry emulsin powder (Kahlbaum) was suspended in 12 cc. of water in a centrifuge tube. After stirring thoroughly, the suspension was centrifuged until the liquid was clear and the supernatant liquid was then decanted and filtered.

To 100 mg. of 6-glucosidogalactose were added 2 cc. of the emulsin solution and 1 drop of toluene. At the same time a tube containing 100 mg. of the disaccharide in 2 cc. of water + 1 drop

of toluene and a tube containing 2 cc. of emulsin solution + 1 drop of toluene were prepared. The solutions were then incubated during 4 days at 37°.

Each solution was now diluted to 200 cc. with water and the reducing power was determined (on 5 cc. samples), a modification<sup>7</sup> of Willstätter's method being used. The emulsin showed a small but definite iodine consumption, which was deducted from the titer of the hydrolyzed disaccharide.

The results showed that the disaccharide was completely hydrolyzed by emulsin under these conditions, indicating that the synthetic disaccharide is a  $\beta$ -glucoside (6- $\beta$ -glucosidogalactose).

*Synthetic 6-Glucosidodulcitol*—The action of emulsin on 6-glucosidodulcitol was investigated as described for the parent synthetic disaccharide.

After treatment with the emulsin solution during 4 days at 37°, the originally non-reducing solution acquired a reducing value corresponding to the liberation of 1 hexose unit from each glucosidodulcitol molecule, indicating that the substance is a  $\beta$ -glucoside.

*Action of Emulsin on Aldobionic Acid (of Gum Arabic) and Its Derivatives. Experiment A, Free Aldobionic Acid*—Addition of a clear, filtered aqueous solution of emulsin to an aqueous solution of the aldobionic acid gave an immediate heavy, white precipitate.

*Experiment B, Sodium Aldobionate*—The calculated volume of sodium hydroxide (0.1 N solution) was added to a sample of crystalline aldobionic acid. Incubation of this solution with clear emulsin solution gave no precipitate but no increase in reducing power was observable even after treatment during 5 days at 37°.

*Experiment C, Methyl Aldobionate*—A specimen of the crystalline methyl ester<sup>8</sup> (m.p. 119°) of the aldobionic acid was similarly incubated with emulsin solution. The mixture was initially clear, but a heavy precipitate gradually developed. After 5 days at 37° no increase in reducing power had developed.

*Experiment D, Methyl Ester of 6-Glucuronidodulcitol*—A specimen of the methyl ester of glucuronidodulcitol (from the aldobionic acid of gum arabic) was incubated with emulsin solution. The

<sup>7</sup> Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

<sup>8</sup> Hotchkiss, R. D., and Goebel, W. F., *J. Biol. Chem.*, **115**, 285 (1936).

mixture was initially clear, but a heavy precipitate gradually formed. After 5 days at 37°, no reducing power had developed.

*Experiment E, 6-Glucosidodulcitol from Aldobionic Acid*—The action of emulsin on this glucosidodulcitol (from the aldobionic acid) was investigated as described for the synthetic disaccharide.

After treatment with the emulsin solution during 6 days at 37°, the originally non-reducing solution acquired a reducing value corresponding to the liberation of 1 hexose unit from each glucosidodulcitol molecule, indicating that the substance is a  $\beta$ -glucoside. It follows that the parent aldobionic acid is a  $\beta$ -glucuronide.

*Experiment F, 6-Glucosido-" $\beta$ "-Methylgalactoside*—The 6-glucosido- $\beta$ -methylgalactoside used in this experiment was prepared as described in the accompanying communication.<sup>1</sup> An aqueous solution of the methylbioside was incubated with emulsin during 5 days at 37°. Determination of reducing sugar by the micro-Willstätter<sup>7</sup> method then revealed the liberation of one CHO group per molecule of methylbioside.

That this scission involved the biose linkage, and not the glycosidic methoxyl group, is shown by Experiment E above.

*Preparation of Crystalline Nonaacetyl 6- $\beta$ -Glucosidodulcitol*—To 1 gm. of dry, recrystallized synthetic 6- $\beta$ -glucosidodulcitol was added a solution of 25 cc. of dry pyridine in 20 cc. of acetic anhydride and the suspension shaken at room temperature until all the solid had dissolved. After standing overnight at room temperature, the solution was poured into 1 liter of filtered ice water, with vigorous stirring. The product separated as a colorless crystalline powder, in quantitative yield. After it was washed with water and dried, it was recrystallized from 15 cc. of absolute ethyl alcohol, giving tufts of colorless long, fine needles having a melting point of 147–148° and the following composition.

5.294 mg. substance: 9.685 mg. CO<sub>2</sub> and 2.725 mg. H<sub>2</sub>O

6.256 " " : 7.79 cc. 0.01 N sodium thiosulfate

C<sub>33</sub>H<sub>42</sub>O<sub>18</sub>. Calculated. C 49.84, H 5.9, COCH<sub>3</sub> 53.61

Found. " 49.89, " 5.8, " 53.54

The substance had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.60^\circ \times 100}{2 \times 1.026} = -29.2^\circ \quad (\text{in acetone})$$

*Preparation of Methylglycoside of Heptamethyl Glucosidogalactose from Synthetic 6-β-Glucosidogalactose*—5 gm. of dry, recrystallized 6-β-glucosidogalactose were dissolved in 30 cc. of water and 20 cc. of acetone were added. The solution was placed in a flask provided with a reflux condenser and an efficient mechanical stirrer, stirred mechanically, and heated in a bath at 35°.

5.5 cc. of dimethyl sulfate were added in one portion, followed by the dropwise addition of 11.7 cc. of 30 per cent aqueous sodium hydroxide during 3 hours, the temperature being raised slowly from 35° to 60°. The solution was then non-reducing to boiling Fehling's solution.

The temperature was now maintained at 60° while 22 cc. of dimethylsulfate and 47 cc. of 30 per cent sodium hydroxide solution were added dropwise during 50 minutes. The temperature was finally maintained at 100° for a further 30 minutes. The solution was cooled to 0° and rendered almost neutral with N sulfuric acid, the neutralization being completed by passing in carbon dioxide.

The solution was extracted repeatedly with chloroform and then the aqueous layer was evaporated to dryness and the solid product extracted several times with boiling chloroform under a reflux condenser. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness under diminished pressure, giving a colorless, viscous syrup (weight 6.0 gm.). The product was now dissolved in 10 cc. of cold acetone and 50 cc. of methyl iodide were added, the solution remaining clear and colorless. 5 gm. of silver oxide were added and the solution was boiled gently under a reflux, with mechanical stirring. Four further portions (5 gm. each) of silver oxide were added at intervals of 30 minutes and the reaction was allowed to proceed for a total of 5 hours.

The product was isolated in the usual manner<sup>9</sup> and remethylated with the same amounts of Purdie's reagents (*without* the addition of acetone).

The resulting fairly mobile syrup (6.5 gm.) was distilled, giving a main fraction boiling at 176° at 0.25 mm. (bath temperature

<sup>9</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **105**, 419 (1934).

196°) and a small second fraction (0.72 gm.) at a bath temperature of 200–225° and pressure of 0.25 mm.

The first fraction, which was mainly the *heptamethyl methylbioside*, crystallized spontaneously.

The second fraction, most of which was *hexamethyl methylbioside*, crystallized in long, fine colorless needles on stirring with dry ether and was recrystallized from ether or ether-pentane. It had  $n_D^{25} = 1.4685$  (superfused substance), a melting point of 119° and the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.26^\circ \times 100}{2 \times 0.864} = -15.1^\circ \quad (\text{in absolute ethanol})$$

The substance had the following composition.

4.784 mg. substance:	9.075 mg. CO <sub>2</sub> and 3.560 mg. H <sub>2</sub> O
3.090 " "	: 29.42 cc. 0.01 N sodium thiosulfate
C <sub>19</sub> H <sub>36</sub> O <sub>11</sub> .	Calculated. C 51.78, H 8.2, OCH <sub>3</sub> 49.32
	Found. " 51.73, " 8.3, " 49.22

The *heptamethyl methylbioside* was recrystallized from pentane (0.4 gm. in 20 cc.), giving rosettes of long, fine colorless needles. It had a melting point of 75–77°,  $n_D^{25} = 1.4635$  (for the superfused substance), and the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.63^\circ \times 100}{2 \times 1.236} = -25.5^\circ \quad (\text{in absolute ethanol})$$

The substance had the following composition.

4.746 mg. substance:	9.210 mg. CO <sub>2</sub> and 3.540 mg. H <sub>2</sub> O
3.300 " "	: 33.75 cc. 0.01 N sodium thiosulfate
C <sub>18</sub> H <sub>32</sub> O <sub>11</sub> .	Calculated. C 52.83, H 8.4, OCH <sub>3</sub> 54.63
	Found. " 52.91, " 8.4, " 52.82

The hexamethyl derivative is very much less soluble in cold ether or pentane than is the heptamethyl derivative. Hence their separation by use of these solvents is readily effected. A sample of crude heptamethyl derivative containing a little hexamethyl methylbioside was purified as follows: 0.5 gm. was dissolved in 10 cc. of dry ether, 30 cc. of pentane were added, and the clear colorless solution kept in the refrigerator overnight. A crop of pure hexamethyl derivative (m.p. 119°) was deposited,

whereas the heptamethyl derivative remained in solution even on addition of a further 70 cc. of pentane.

*Simultaneous Catalytic Reduction and Deacetylation of Methyl Ester of Heptaacetyl Aldobionic Acid*—2.5 gm. of crystalline heptaacetyl 6- $\beta$ -glucuronidogalactose methyl ester<sup>8</sup> were dissolved in 100 cc. of absolute methanol. 2.5 gm. of copper chromite catalyst were added and the mixture was shaken in an atmosphere of hydrogen at a pressure of 3300 pounds per sq. inch during 5 hours at 175°.

The product was isolated in the usual manner, giving 1.3 gm. of a colorless flaky glass which contained no uronic acid (naphthoresorcinol test) and was non-reducing to boiling Fehling's solution, but was strongly reducing after hydrolysis by dilute mineral acid. It was readily soluble in water. These properties suggest the formation of glucosidodulcitol but as the crude material was impure and difficult to purify, it was not further investigated.





## PROTEIN MONOLAYERS: FILMS OF OXIDIZED CYTOCHROME C

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The intracellular respiratory pigment, an enzyme known as cytochrome, discovered by MacMunn (1) in 1886, is found not only in the muscles and other tissues of almost every order of the animal kingdom, but also in plants, and in the cells of unicellular organisms, such as bacteria and yeast. Thus it is a very widely distributed pigment of aerobic organisms and as an iron-porphyrin system of compounds belongs to the same family as hemoglobin. The main respiratory system of the types of cells mentioned above is supposed to consist of indophenol oxidase, cytochrome C (and probably A and B), Szent-Györgyi's dicarboxylic acid catalysts, di- or triphosphopyridine nucleotides, and dehydrogenases and their substrates, such as *Zwischenferment* and hexosemonophosphate. Even when the oxidation chain differs from this, the presence of cytochrome C seems essential.

The relations of cytochrome outlined above indicate the importance of any investigation which reveals some of its unknown characteristics. Since very recently cytochrome C, the most stable of the three hemochromogens designated as cytochromes A, B, and C, has been isolated by Keilin and Hartree (2), it has become possible to determine the surface pressure-area relations of the pure compound on aqueous subphases.

As a protein cytochrome C is of special interest, since it has a high isoelectric point at a pH of 9.9 or 10.0. According to Zeile (3) the cytochrome hemin group of this protein differs from protoporphyrin in being condensed with a tertiary nitrogenous ring base, which may account for its highly basic character.

We are greatly indebted to Dr. E. Stotz for a generous supply

of cytochrome C from material which he prepared in the course of an investigation of the spectroscopic properties of the material under the direction of Professor T. R. Hogness.

*Preparation of Solutions and Experimental Technique*—The cytochrome C used was isolated from beef heart muscle by the method of Keilin and Hartree (2) and its purity tested by Dr. Stotz. It contained 0.34 per cent iron determined by the method of Lintzel (4) modified to the extent of measuring the pink  $\text{Fe}^{++}$ -dipyridyl complex spectrophotometrically at  $\lambda = 5200 \text{ \AA}$ . From the iron analysis the original standard solution (in 1 per cent sodium chloride) contained  $1.136 \times 10^{-7}$  moles of iron (or cytochrome) per cc. Spectrophotometric estimation of the reduced form at  $\lambda = 5497 \text{ \AA}$ . by the method of Keilin and Hartree (2) showed that it contained  $1.14 \times 10^{-7}$  moles per cc. For the oxidized form Theorell (5) gave the specific molal absorption coefficient as  $2.75 \times 10^7$  cc. per mole at  $\lambda = 5300$ , while the value  $2.69 \times 10^7$  was obtained for the material used.

The standard solution prepared by Dr. Stotz was made to contain 3 per cent of alcohol, and definite volumes of this solution were allowed to spread over the subphase solution in the trough of a film balance of a type similar to that already described in a publication from this laboratory (6).

Buffer subphase solutions were made up from carbonate and bicarbonate to be in approximate equilibrium with the carbon dioxide in the air. The pH of the subphase was considered to be that of the bulk solution as measured with a glass electrode after it had come to equilibrium with the carbon dioxide in the air. The accuracy was about 0.1 pH unit. The results obtained in this way were self-consistent. Solutions of  $\text{pH} > 10.5$  were made of sodium hydroxide and the pH taken to be that of the bulk solution, while in acid subphases hydrochloric acid was used. The buffers used at each pH below 10.5 are listed in Table I.

After the cytochrome had been spread on these dilute solutions of the buffers, 3 minutes being allowed for this process, force-area curves were determined by advancing the barrier of the film balance every minute and measuring the surface pressure 45 seconds after each compression. The same technique was employed in each series of determinations in order to standardize as far as possible the errors due to solution and collapse of the films.

Surface pressures could be determined to  $\pm 0.05$  dynes per cm. Specific areas are probably accurate to 1 per cent, while relative values of the specific areas are probably good to 0.3 per cent.

*Pressure-Area Relations and Effects of Hydrogen Ion Concentration (pH)*—The effects of pH upon the pressure-area relations of cytochrome C are exhibited in Fig. 1, A for values below, and in Fig. 1, B for those above the isoelectric point. It may be noted from the form of the curves that above 10 dynes, and more particularly above 12 dynes of pressure per cm., the films are subject to a lesser or greater degree of collapse. In papers which deal with the collapse of monolayers of any type it is frequently stated that the phenomenon is governed largely by accident. This means that the rapidity of compression, the pH, the temperature, the presence of impurities, etc., affect the collapse. Since so many variables are

TABLE I  
*Composition and Final pH of Buffer Solutions Used*

Buffer or acid	pH
HCl.....	5
$4 \times 10^{-4}$ M NaHCO <sub>3</sub> .....	7.7
$3 \times 10^{-3}$ " ".....	8.4
$3 \times 10^{-3}$ " " $10^{-4}$ M NaOH, $10^{-4}$ M NH <sub>4</sub> OH.....	8.8
$1 \times 10^{-2}$ " " $2 \times 10^{-3}$ M Na <sub>2</sub> CO <sub>3</sub> .....	9.4
$10^{-2}$ M K <sub>2</sub> CO <sub>3</sub> .....	10.0

involved, it is not always possible for the investigator to keep account of all of them. Thus the portions of the curves which exhibit the collapse of the films of oxidized cytochrome C are significant only when the conditions during the collapse, particularly the rapidity of the compression, are taken into account.

At pH 8.8 a duplicate force-area curve was determined after the subphase had been made  $10^{-6}$  M in aluminum chloride, enough aluminum to alter radically the pressure-area curve of stearic acid at this pH. However, with the cytochrome the curves obtained with and without aluminum ions were identical within experimental error at pressures below collapse. In the case of this protein the carboxyl groups, which in stearic acid films are very tightly bound together by adsorbed aluminum ions, are probably held too far apart by the configuration of the protein molecule

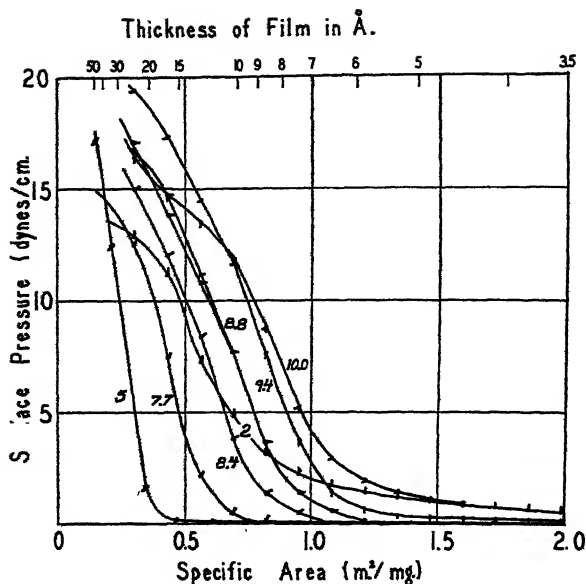


FIG. 1, A. Effect of pH upon pressure-area relations of cytochrome C for values below the isoelectric point.

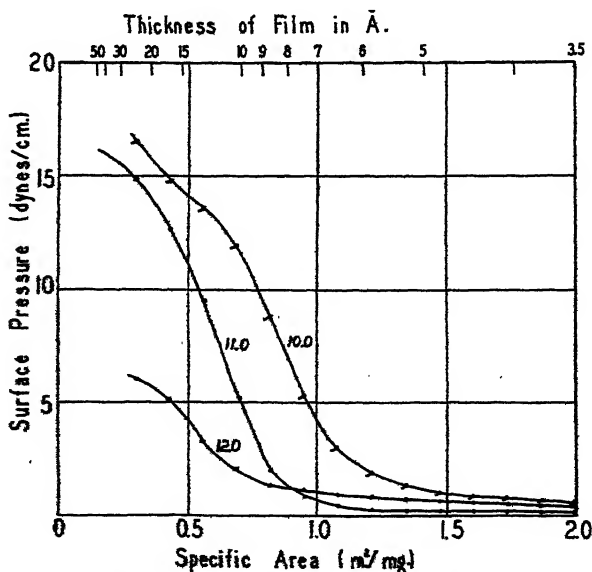


FIG. 1, B. Effect of pH upon pressure-area relations of cytochrome C for values above the isoelectric point.

for an aluminum ion to form a bond with more than one group. It would be interesting to determine the effect of the carboxyl groups and amino groups in the protein at each pH by determining the amount of absorption of aluminum and of copper ions by the film.

The variation of the specific area, and of the thickness of the film, with pH is exhibited in Fig. 2. In the calculation of the thickness of the film the specific volume of the protein was assumed to be 0.707 cc. per gm.

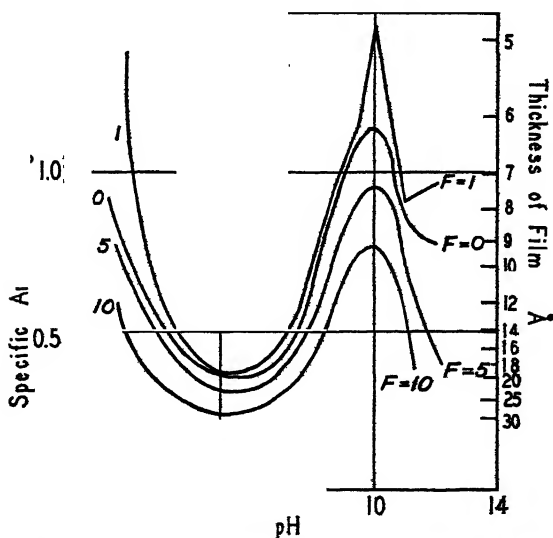


FIG. 2. Variation of specific area and of thickness of the film with pH.  $F$  represents surface pressure.

The work described in this paper reveals no property of the film which is governed by the active hemin group, so it would be important to determine whether the reduced form of cytochrome exhibits any such property.

**Thickness of Films**—From Fig. 2 it is apparent that the thinnest films of oxidized cytochrome C are obtained at the isoelectric point, where the thickness is about 3.5 Å. at 0.5 dyne per cm. and 9.3 Å. at 10 dynes per cm. Thicknesses below about 4.5 Å. indicate that the entire surface of the liquid is not covered by the

protein, though the holes may be merely those due to the non-fitting of the intramolecular structure of the protein.

One of the most striking characteristics revealed by Figs. 1, *A* and 1, *B* is that either the protein film is very much more compressible, or possibly more soluble, at a pH (2) very far below the isoelectric point, or at one (12) considerably above this point. This is explained by the relations of the absorption spectra. Thus Theorell finds that the red solution of oxidized cytochrome C becomes brown at a pH below 2.5 and that the absorption spectrum also changes if the pH is raised to a very high value, as 13. With reduced cytochrome and carbon monoxide a remarkable change is found by Altschul and Hogness (7) when the pH is lowered to 3.8. That is, at this value the Soret band  $\lambda$  4140 Å. increases in intensity by 56 per cent, and at a pH of 2 the intensity increase is 351 per cent. On the alkaline side there is an increase of 55 per cent at a pH of 12, and almost the same, 56 per cent, at a pH of 13. The absorption spectra are almost the same in form at the low and high pH, but different from that at any intermediate value.

Theorell considers that oxidized cytochrome C exists in three forms, the acid form, the intermediate or ordinary form, and the alkaline form. According to Keilin (8) the reduced form is autoxidizable in the pH region of either the acid or the alkaline form of oxidized cytochrome C. At room temperature Theorell found the autoxidation to be completed in 4 minutes at pH 3, but in a twenty-fourth of this time at pH 2.

Thus the high compressibility, or solubility, of the film at high and low pH, that is, at 2 and 12, may be related to the fact that the form of the cytochrome is not the same as at intermediate values.

At the low pH the change from the normal to the abnormal form is reversible, and the cytochrome acts as an acid-base indicator, with a dissociation constant, as given by Theorell, of  $10^{-3}$ . The change at the high pH is also reversible, and the dissociation constant is of the order of  $10^{-12}$ .

It is obvious from what is stated above that the pressure-area relations of cytochrome C change very rapidly in the regions of pH in which these reversible changes occur.

In the work described in this paper, as in that of Gorter and

coworkers (9) on proteins in general, not much time was allowed for completion of the spreading of the protein. In such a case the film is thinnest at the isoelectric point, where, according to Philippi (10), the monolayer spreads most rapidly. According to him lactoglobulin, insulin, and similar proteins, give, after complete spreading, their thickest films at this point.

The behavior of films at the isoelectric point, as contrasted with other values for the pH, is presumably related to the following characteristics of the protein at this point: (1) the concentration of the zwitter ions is greater than for any other pH, and (2) the number of positive ions of the protein is equal to the number of negative ions.

In the next paper of this series we will consider the variation of the thickness of monolayers of cytochrome C with (1) the time allowed for spreading, and (2) the concentration of positive and negative mono- and bivalent ions of salts in the subphase.

#### SUMMARY

Monolayers of cytochrome C, a substance which acts as an extremely stable protein and in certain respects as an enzyme, are found to exhibit on water thicknesses from 3.5 Å. at a film pressure of 0.5 dyne per cm. and a pH of 10, to 50 Å. at 18 dynes per cm. and pH 5.

The thinnest films were obtained at the isoelectric point and the thickest at pH 5. Further work on the effects of time and of the ion concentration of the subphase is needed before the theory of the behavior of the spreading of the films is discussed in detail, but it seems probable that the change in spreading with pH is influenced largely by the relative concentrations of positive, negative, and zwitter ions of the protein, and by the ions of salts in the subphase.

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## SOLUBILITY OF BONE SALT

### II. FACTORS AFFECTING ITS FORMATION

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On the basis of analytical data alone, it was assumed for many years that the bone salt consisted of a mixture of salts such as  $\text{Ca}_3(\text{PO}_4)_2$  or hydroxyapatite and  $\text{CaCO}_3$  (1-3). At the same time, those who chose to ignore the variations in the composition of different samples of bone proposed that it consisted of a definite single chemical individual (4-6).

Following the demonstration by x-ray diffraction patterns that the bone salt is not a mechanical mixture but is a compound belonging to the apatite series (7), there was a tendency to revert to the ideas implied in the thesis that bone is a single chemical individual. It was, therefore, proposed that the bone salt had a definite solubility product and its precipitation or solution was determined by the activity of its constituent ions in solution (8). This point of view ignored the fact, long known (1), that in young bone the proportion of  $\text{CO}_2$  to  $\text{PO}_4$  is much less than in adult bone. It also cannot be reconciled with the recent observations (9) that the ion product  $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{--}]^2$  in a liquid phase in equilibrium with the bone salt varies with the amount of solid phase present, and that the ion product required to initiate precipitation is very much greater than that required to initiate solution.

Any satisfactory account of the formation of the bone salt must, therefore, be consistent with these observations.

It was to provide further details regarding the intimate changes which take place during the formation of the bone salt that the present experiments were devised and carried out. Specifically, they were planned to answer the following questions. (1) What is the initial aggregation of atoms formed from solution? (This

question has not been directly answered by experiment, but evidence will be presented in support of the hypothesis that it contains calcium and phosphate in the ratio of 1:1.) (2) What is the first crystalline precipitate formed? (Evidence will be presented that it contains calcium and phosphate in the ratio of 3:2.) (3) How does the precipitate increase in size? (Evidence will be presented that it increases by removal of calcium and phosphate from solution in the ratio of 1:1, followed by the loss of phosphate from the precipitate.) (4) What changes take place in the precipitate with the passage of time? (Evidence will be presented that, if the solution contains  $\text{HCO}_3^-$ , the precipitate removed Ca and  $\text{CO}_2$  from solution below the solubility product of  $\text{CaCO}_3$ . If no  $\text{CO}_2$  is present, Ca and OH are removed by the precipitate. The removal of these ions (or those of other calcium compounds present in solution in amounts approaching their solubility products) represents the process by which the precipitate changes in composition.)

### *Procedure*

In order to follow the changes which took place in the solid phase, the changes occurring in the liquid phase were determined at intervals during the precipitation of calcium phosphate in three types of solutions: (1) those containing bicarbonate, at constant pH 7.3 and phosphate in excess of the calcium (the amount of phosphate was varied in individual experiments); (2) those containing bicarbonate and a constant excess of phosphate at pH 7.0 and pH 7.4; (3) those containing no bicarbonate, at constant pH, with calcium equivalent to and in excess of the phosphate.

The details of the experimental procedures employed in the preparation of the precipitates and the analytical data from which subsequent calculations were made follow.

*Preparation of Carbonate-Containing Precipitates in Solutions of Varying Phosphate Content*—In three flasks, calibrated to contain 1100 cc., the amounts of  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{HPO}_4$ , and  $\text{NaCl}$  presented in Table I were added at  $38^\circ$ .  $\text{HCl}$  was added to bring the pH to 7.3 and distilled water to make the volume 60 per cent of the volume of the flask. The recorded amount of  $\text{CaCl}_2$  dissolved in a volume of water equal to 30 per cent of the volume of the flask was then added while the solution was rapidly stirred

by means of a mechanical stirrer. When the pH fell below 7.3, observed colorimetrically, the addition of  $\text{CaCl}_2$  was stopped and  $\text{NaOH}$  was added to bring it back to the original value. The addition of the  $\text{CaCl}_2$  in each of the experiments (Table I) occupied 20 minutes.

Distilled water was added to bring the solution to volume, and after being stoppered and rotated for 5 minutes, a sample was removed for analysis and centrifuged under oil. To prevent

TABLE I

*Changes in Composition of Liquid Phase (in mm per Liter) during Precipitation of Calcium Phosphate from Solutions Containing Bicarbonate with Varying Phosphate, pH Constant*

The solutions contained  $10^{-4}$  per cent phenol red.  $\text{NaOH}$  was added to maintain the pH at 7.3 for 12 days. The pH determined by means of the glass electrode at 20 days was 7.17 in Experiment 117, 7.22 in Experiment 122, and 7.17 in Experiment 115.

Ex- peri- ment No.	Reagents added		Composition of liquid phase					
			5 min.		24 hra.	4 days	8 days	20 days
117	$\text{Na}_2\text{HPO}_4$	40.9	Ca P $\text{CO}_2$	0.409	0.278			0.0542
	$\text{NaHCO}_3$	22.28		3.75	5.46	5.65	5.73	5.78
	$\text{NaCl}$	58.1		18.50	17.21	17.08	17.04	17.04
	$\text{CaCl}_2$	54.54						
112	$\text{Na}_2\text{HPO}_4$	54.54	Ca P $\text{CO}_2$	0.162	0.120			0.065
	$\text{NaHCO}_3$	22.28		15.35	17.30	18.30	18.40	18.40
	$\text{NaCl}$	40.9		20.10	18.61	18.65	18.45	18.45
	$\text{CaCl}_2$	54.54						
115	$\text{Na}_2\text{HPO}_4$	81.8	Ca P $\text{CO}_2$	0.14	0.117			0.076
	$\text{NaHCO}_3$	22.28		41.6	41.9	44.10	44.8	46.1
	$\text{NaCl}$	0		20.90	19.96	19.35	19.10	18.79
	$\text{CaCl}_2$	54.54						

the escape of  $\text{CO}_2$ , molten paraffin (m.p.  $44^\circ$ ) was added to fill the space above the liquid in the flask. The paraffin was solidified and the flask was rotated continuously at  $38^\circ$ . Analysis of the liquid was made after 24 hours, 4 days, 8 days, and 20 days. The spaces occupied by the samples were filled with molten paraffin. The total volume of  $\text{NaOH}$  added, subsequent to the removal of the first sample, was less than 2 per cent of the final volume of the solution.

*Preparation of Carbonate-Containing Precipitates in Solutions of Varying pH*—Inasmuch as it is difficult to keep the pH entirely constant during the precipitations, experiments were designed to determine the effect of changing the pH from 7.0 to 7.4. For this purpose, the reaction vessel was arranged so that samples could be removed at frequent intervals without disturbing the rotation of the main portion. The solutions were admitted to a flask equipped with a mechanical stirrer, which operated through a mercury seal. The solution was covered with a thick layer of paraffin oil. To 2 liters of solution, containing 225 mm of  $\text{Na}_2\text{HPO}_4$ , 30 mm of  $\text{NaHCO}_3$ ,  $10^{-4}$  per cent phenol red, and HCl to bring the reaction approximately to pH 7.0 or 7.4, was added 1 liter of solution containing 315 mm of  $\text{CaCl}_2$  and  $10^{-4}$  per cent phenol red. The pH during the addition was controlled colorimetrically by comparison with 0.05 M phosphate buffer solution containing  $10^{-4}$  per cent phenol red and NaCl to increase  $\mu$  to 0.2. The buffer solution was contained in a vessel similar to that used for precipitation. When the pH of the precipitation mixture dropped below that of the control flask, the addition of  $\text{CaCl}_2$  was stopped and approximately 0.5 N NaOH was added to bring it back to the original value. The solution was continuously and rapidly stirred. The amounts of reagents added per liter of the final solution are given in Table II. Samples for analysis were withdrawn at frequent intervals during the first 24 hours of equilibration. The pH of each sample was determined by means of the glass electrode, and the pH of the solution adjusted as nearly as possible to the original value. To obtain the final points, a flask was filled with a uniform portion of the mixture, stoppered, and rotated. The solution of Experiment 10 was maintained near pH 7.4; that of Experiment 12 was maintained near pH 7.0. Analyses of the liquid phase are shown in Table II. In these experiments, the total volume of the solutions was calculated from the sum of the volumes of the reagents added. It was considered advisable to resort to such an approximation in order to use an apparatus from which samples could be quickly withdrawn without disturbing the rotation of the main portion. These results were useful for comparing the difference in  $\text{CO}_2$  uptake and phosphate loss by the precipitate at different pH values of the solution during equilibration.

*Preparation of Precipitates from Solutions Containing Excess and Equivalent Amounts of Calcium*—To prevent the precipitation of calcium carbonate as a separate solid phase, the carbonate-containing precipitates described in the foregoing section were pre-

TABLE II

*Composition of Liquid Phase during Precipitation of Calcium Phosphate from Solutions Containing Bicarbonate, pH Varied*

$\text{CaCl}_2$  was added to a mixture of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaHCO}_3$  in mm per liter of final solution in the following amounts: Experiment 10,  $\text{CaCl}_2$  95.0,  $\text{Na}_2\text{HPO}_4$  67.9,  $\text{NaHCO}_3$  9.05; Experiment 12,  $\text{CaCl}_2$  97.4,  $\text{Na}_2\text{HPO}_4$  69.6,  $\text{NaHCO}_3$  9.28.

Additions of the  $\text{CaCl}_2$  were carried out at the rate of 75 cc. per minute.  $\text{NaOH}$  was added when the pH fell below 7.4 (Experiment 10) or 7.0 (Experiment 12). The total volume of  $\text{NaOH}$  added per liter of solution after removal of the first sample was 9 cc. (Experiment 10) and 8 cc. (Experiment 12). The solutions contained  $10^{-4}$  per cent phenol red.

Experiment 10					Experiment 12				
Time*	pH	Ca	P	$\text{CO}_2$	Time*	pH	Ca	P	$\text{CO}_2$
hrs.		mm per l.	mm per l.	mm per l.	hrs.		mm per l.	mm per l.	mm per l.
0.2	7.60	0.882	2.25	5.12	0.25	7.05	2.23	2.56	6.0
0.75	7.45	0.344	3.08	4.94	0.75	7.12	1.62	2.61	5.79
2.5	7.47	0.264	3.86	4.53	1.25	7.05	1.25	2.82	5.38
4.0	7.41	0.244	4.10	4.24	1.75	6.96	1.18	2.96	5.19
5.0	7.42	0.208	4.20	4.09	2.25	6.89	1.42	3.32	4.95
9.0	7.51		4.62	4.08	3.25	6.84	1.49	3.68	4.76
days									
1	7.35	0.136	4.81	3.99	4.25	6.84	1.22	3.96	4.46
3	7.37		5.30	3.85	5.25	6.91	0.65	3.96	4.46
10	7.22	0.1067	5.40	3.84	8.25	6.90		4.48	3.81
					days				
					1	6.85		5.34	3.66
					2	7.04		5.32	3.50
					28	6.92	0.74	6.13	3.04

\* After initial mixing of reagents.

pared from solutions which contained an excess of phosphate. In order to follow the course of precipitation when the calcium is equivalent to or in excess of the phosphate, two precipitates were prepared in solutions free from  $\text{CO}_2$ . For this purpose, a solution

of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaOH}$  was added to an excess of  $\text{CaCl}_2$  (Experiment 130); and in a second experiment (No. 131), a solution of  $\text{CaCl}_2$  was added to an equivalent quantity of  $\text{Na}_2\text{HPO}_4$  plus  $\text{NaOH}$  to maintain the pH at  $7.3 \pm 0.1$  (Experiment 131). The amounts of the reagents employed and analyses of the solutions after varying elapsed times are given in Table III.

TABLE III

*Changes in Composition of Liquid Phase When Calcium Phosphate Was Precipitated from Solutions Containing Excess Calcium (Experiment 130) and Equivalent Amounts of Calcium and Phosphate (Experiment 131)*

In Experiment 130, 50.0 mm of  $\text{Na}_2\text{HPO}_4$  and 39.7 mm of  $\text{NaOH}$  dissolved in 600 cc. were added during 2 minutes to 80.0 mm of  $\text{CaCl}_2$  dissolved in 300 cc. Additional  $\text{NaOH}$  was added to maintain the pH at 7.3. The solution was made to a volume of 1000 cc.

In Experiment 131, 75.0 mm of  $\text{CaCl}_2$  dissolved in 300 cc. of solution were added during 2 minutes to 50.0 mm of  $\text{Na}_2\text{HPO}_4$  and 26.8 mm of  $\text{NaOH}$  dissolved in 300 cc. Additional  $\text{NaOH}$  was added to maintain the pH at 7.3. The solution was made to a volume of 1000 cc.

The pH was not adjusted during the last week in either experiment, and as determined by the glass electrode at 30 days was 6.74 in Experiment 130 and 7.06 in Experiment 131.

The solution contained  $10^{-4}$  per cent phenol red.

Experiment No.	Time	Ca	P	Experiment No.	Time*	Ca	P
		mm per l.	mm per l.			mm per l.	mm per l.
130	2 min.	8.99	0.395	131	2 min.	7.32	0.606
	30 "	6.58	0.053		10 "	4.99	0.192
	4 hrs.	3.55	0.021		68 "	1.26	0.032
	24 "	2.31	0.019		19 hrs.	0.52	0.046
	30 days	0.74	0.046		30 days	0.10	1.06

\* After completion of addition of calcium and phosphate.

*Procedure for Determination of Composition of Precipitates*—For the purpose of determining the composition of the precipitates at a given time, it was found necessary, in these experiments, to calculate the results from the difference between the known amount of reagents added and the composition of the liquid phase at a given time. This was necessary because the composition

of the precipitate constantly changes when it is in contact with the liquid phase.

The experiments were conducted in such a way as to decrease, as far as possible, the errors of the procedure adopted. For that purpose, the amounts of solid (18 to 30 mm calculated as  $\text{Ca}_3(\text{PO}_4)_2$ ) precipitated per liter of solution were made as large as practicable. The largest error likely to be encountered in the calculation of the composition of the solid from the composition of the liquid phase occurs when the liquid contains phosphate or calcium in amounts approaching the amount contained in the precipitate. For instance, in Experiment 115, Table I (highest phosphate), an error of 1 per cent in the phosphate analysis of the liquid would make an error of 2 per cent in the phosphate content of the precipitate. Therefore, the phosphate content of this precipitate is not included in the calculations. The precipitates contain sodium, the amount of which cannot be determined except in washed precipitates, a procedure which in itself changes the composition of the solid. (The experimental evidence for this will be presented in a succeeding paper.) For the purpose of calculation of the composition of the precipitate, the sodium content is assumed to be 2.3 per cent of the calcium, which is similar to that found after washing similarly prepared precipitates free from chloride. The actual sodium content of the precipitates is undoubtedly higher, and the error caused is in the direction of making the precipitates appear to contain more  $\text{CaHPO}_4$  than is actually the case. It is unlikely that the original sodium content of the precipitates is greater than the sodium and potassium content of the inorganic portion of bone. If such is the case, the error introduced does not exceed 1 per cent.

Calcium was determined by the method of Fiske and Logan (10); phosphate by the method of Fiske and Subbarow (11); carbon dioxide by the procedure of Van Slyke and Neill (manometric) (12); pH with a MacInnes glass electrode and a Leeds and Northrup potentiometer-electrometer.

### *Symbols and Calculations*

The composition of the precipitates reported in Table IV was calculated from the initial and final composition of the liquid phase as follows:



- (1)  $(Ca)_p = (Ca)_i - (Ca)_l$   
 where  $(Ca)_p$  = mm total Ca in the precipitate  
 $(Ca)_i$  = " " " present in the original mixture  
 $(Ca)_l$  = " " " " " liquid after the precipitate formed
- (2)  $(CO_2)_p = (CO_2)_i - (CO_2)_l$   
 where  $(CO_2)_p$  = mm  $CO_2$  in the precipitate = mm  $CaCO_3$   
 $(CO_2)_i$  = " " " " original mixture  
 $(CO_2)_l$  = " " " " liquid after the precipitate formed
- (3)  $(P)_p = (P)_i - (P)_l$   
 where  $(P)_p$  = mm P in the precipitate  
 $(P)_i$  = " " " " original mixture  
 $(P)_l$  = " " " " liquid after the precipitate formed
- (4)  $(HPO_4)_p + (PO_4)_p = (P)_p$   
 where  $(HPO_4)_p$  = mm  $HPO_4$  in the precipitate = mm  $CaHPO_4$  + mm  $Na_2HPO_4$   
 $(PO_4)_p$  = "  $PO_4$  in the precipitate =  $2 \times$  mm  $Ca_3(PO_4)_2$  + mm  $Na_3PO_4$
- (5)  $2(HPO_4)_p + 3(PO_4)_p = (TB)_p - 2(CO_2)_p$   
 where  $(TB)_p$  = m.eq. total base in the precipitate  
 $(TB)_p = 1.023 \times 2(Ca)_p$
- (6)  $2(Ca(OH)_2)_p = (TB)_p - 3(P)_p - 2(CO_2)_p$   
 where  $(Ca(OH)_2)_p$  = mm  $Ca(OH)_2$  in the precipitate

### Results

*Composition of Precipitates*—The composition of four precipitates soon after addition of the reagents and after long equilibration is given in Table IV.

The results of the calculations indicate that within a few minutes after the reagents are added together, more than 75 per cent of the calcium of the precipitate was united with the phosphate in the ratio corresponding to  $Ca_3(PO_4)_2$ .<sup>1</sup> The calculated proportion corresponding to this ratio became progressively greater during

<sup>1</sup> As a convenient means of notation, increases in  $CO_2$  and equivalent amounts of Ca in the precipitate are referred to as increases of  $CaCO_3$ . It is presumed, however, that the Ca and  $CO_2$  do not exist in the crystal as a separate phase. Ca and  $PO_4$  in the ratio 3:2 in the precipitate are denoted as  $Ca_3(PO_4)_2$ , although, as will be shown, such a substance has the capacity to add or include additional Ca and  $CO_2$  or OH, etc., in its crystal structure. Phosphate calculated to be in excess of the ratio 3Ca:2P is denoted as  $CaHPO_4$ . Ca in excess of the anions known to be present in the precipitate is referred to as  $Ca(OH)_2$ . Such a notation is considered expedient, because the amount of Ca and  $CO_2$  or OH, etc., taken up by the precipitate varies with the concentration of those ions in the liquid phase.

the contact of the precipitate with the liquid phase. Likewise, the calculated  $\text{CaCO}_3$  content of the precipitates increased during equilibration. When the precipitates were formed in solutions containing no  $\text{CO}_2$  but equivalent amounts of calcium and phosphate (i.e., 1.5 moles of Ca to 1 mole of P, Experiment 131, Table IV), or excess calcium (Experiment 130), the precipitates after long equilibration had a composition indicating that they contained  $\text{Ca}(\text{OH})_2$ .

TABLE IV

*Composition of Precipitates Calculated from Amount of Reagents Added and Composition of Liquid Phase*

Experiment No.	Ca added per mole phosphate	Per cent of total base* of ppt. combined as							
		$\text{Ca}_3(\text{PO}_4)_2$		$\text{Ca}(\text{OH})_2$		$\text{CaHPO}_4$		$\text{CaCO}_3$	
		A	B	A	B	A	B	A	B
	<i>moles</i>								
112	1.00	76.8	84.9	0	0	19.3	8.3	3.92	6.86
117	1.33	78.1	82.5	0	0	15.1	8.06	6.8	9.4
131	1.50	83.5	97.0	0	3.0	16.5	0	0	0
130	1.59	92.6	93.6	0	6.4	7.4	0	0	0

A = 5 minutes (Experiments 112 and 117) or 2 minutes (Experiments 130 and 131) after addition of the reagents; B = 20 days (Experiments 112 and 117) or 30 days (Experiments 130 and 131).

\* Strictly,  $\text{Ca}_3(\text{PO}_4)_2$  is the per cent of total phosphate present as  $\text{PO}_4$  and includes a small amount of  $\text{Na}_2\text{PO}_4$ ;  $\text{CaHPO}_4$  is the per cent of total phosphate present as  $\text{HPO}_4$  and includes a small amount of  $\text{Na}_2\text{HPO}_4$ . 22.28 mm of  $\text{NaHCO}_3$  were added per liter in Experiments 112 and 117; the solutions in Experiments 131 and 130 contained no  $\text{NaHCO}_3$ . Details concerning the formation of these precipitates are given in Tables I and III and in the text.

*On Relation between Amounts of Ca and  $\text{CO}_2$  Gained and Phosphate Lost by Precipitates after 5 Minutes Equilibration*—The amounts of Ca and  $\text{CO}_2$  gained and phosphate lost by the precipitates between 5 minutes and 20 days equilibration (Experiments 112, 115, 117), or 2 minutes and 30 days equilibration (Experiments 130 and 131), are shown in Fig. 1.

The results show that the precipitates removed Ca and  $\text{CO}_2$  (or OH presumably, if no  $\text{CO}_2$  was present) from solution. When the precipitates were formed in the presence of excess phosphate,

the Ca which united with the  $\text{CO}_2$  during that time was derived chiefly from loss of phosphate by the precipitate. When the precipitates were formed in solutions containing equivalent amounts of calcium and phosphate, or excess Ca (and no bicarbonate), the amount of phosphate gained or lost by the precipitate was insignificant in relation to the amount of Ca removed from solution.

During this time, therefore, the changes in the precipitate consisted essentially of an increase of  $\text{CaCO}_3$  or  $\text{Ca}(\text{OH})_2$  content and

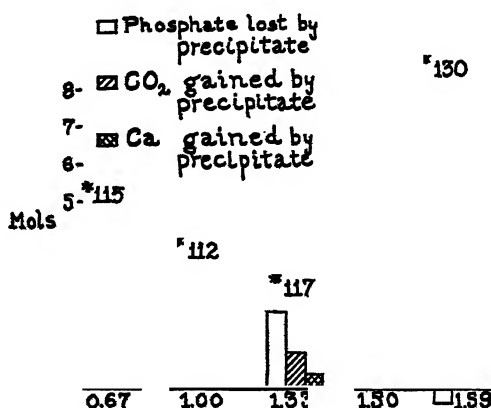


FIG. 1. Relation between gain of Ca and  $\text{CO}_2$  and loss of phosphate during the equilibration of calcium phosphate precipitates. The solution from which precipitates in Experiments 115, 112, and 117 were formed contained 22 mm of  $\text{NaHCO}_3$  per liter; the precipitates from Experiments 130 and 131 contained no  $\text{NaHCO}_3$ . The figures at the bottom of the chart represent the moles of Ca added per mole of phosphate.

(from precipitates formed from excess phosphate) loss of phosphate. Increase in the amount of  $\text{Ca}_3(\text{PO}_4)_2$  in the precipitate or crystal growth was confined essentially to that resulting by loss of phosphoric acid from  $\text{CaHPO}_4$  previously taken up by the precipitate. The phosphate thus lost contributed about equally to crystal growth and  $\text{CaCO}_3$  accretion.

*On Removal of Ca and  $\text{CO}_2$  from Solution by Precipitate*—It was found that  $\text{Ca}^{++}$  and  $\text{CO}_3^{--}$  were removed from solution even though the product  $[\text{Ca}^{++}] \times [\text{CO}_3^{--}]$  was maintained below the solubility product of  $\text{CaCO}_3$ . The ion products expressed as

their negative logarithm,  $p [\text{Ca}^{++}] [\text{CO}_3^{--}]$ , of the first samples of the liquid phases obtained after addition of the  $\text{CaCl}_2$  and  $\text{NaOH}$  to the  $\text{Na}_2\text{HPO}_4$  in Experiments 10, 12, 117, and 115 (Tables I and II) were, respectively, 7.57, 7.61, 7.58, and 7.97. After long equilibration, they were respectively 8.92, 8.53, 8.63, and 8.44. The solubility product of  $\text{CaCO}_3$ , expressed as  $pK_{\text{CaCO}_3}$ , is 7.3 at the corresponding ionic strength. It is obvious that the solubility product of  $\text{Ca}(\text{OH})_2$  was not even closely approached in our experiments, although the precipitates in Experiments 130 and 131 removed  $\text{Ca}$  (and presumably  $\text{OH}$ ) from the solutions.

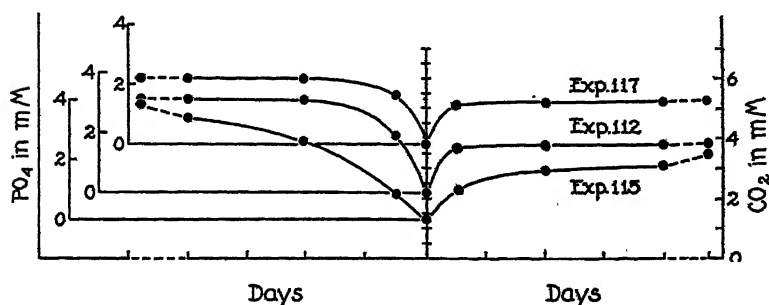


FIG. 2.  $\text{CaCO}_3$  absorbed and phosphate given up by 20 mM of  $\text{Ca}_3(\text{PO}_4)_2$  precipitated in 1.10 liters of solution containing 22.28 mM of  $\text{CO}_2$  per liter at pH 7.3 and  $37.5^\circ$ . The ratio of calcium added to phosphate, in moles, is as follows: Experiment 117, 1.33:1.00; Experiment 112, 1.00:1.00; Experiment 115, 0.67:1.00. The  $\text{Ca}$  taken up by the precipitate after 5 minutes equilibration was 0.35 mM in Experiment 117, 0.10 mM in Experiment 112, and 0.06 mM in Experiment 115. Phosphate given up to the solution after 5 minutes equilibration is shown by the curves to the left of the center. Total  $\text{CO}_2$  taken up by the precipitate is shown by the curves to the right of the center.

*On Relative Amounts of  $\text{CO}_2$  Taken Up by Precipitates Formed in Presence of Different Amounts of Phosphate*—The effect of varying the ratio of phosphate to calcium, in the solution from which the precipitate was formed and subsequently equilibrated, on the rate of  $\text{CO}_2$  gain by and phosphate loss from the precipitate is shown in Fig. 2. It will be observed that the uptake of  $\text{CO}_2$  was greatest from the solution in which the ratio of phosphate to calcium was least; i.e., 1.0:1.33. Since the  $\text{PO}_4^{--}$  activity in this solution was least, the  $\text{Ca}^{++}$  activity was greatest. The

It may be calculated, therefore, that the amount of  $\text{CaCO}_3$  taken up by the precipitate is related to the ion product  $[\text{Ca}^{++}] \times [\text{CO}_3^{--}]$  in the solution, even though the product is considerably less than the solubility product of  $\text{CaCO}_3$ . An exact direct relationship is not to be expected, because the amount of  $\text{CaCO}_3$  taken up is also related to the amount of calcium liberated by loss of phosphate from the precipitate.

In the same way, the difference in final composition between the precipitates in Experiments 130 and 131 (Table IV) shows

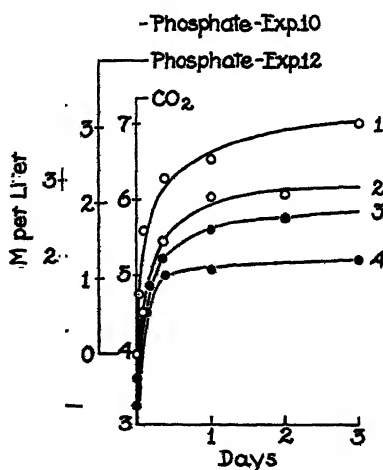


FIG. 3. Changes in the liquid phase during the precipitation of calcium phosphate in solutions containing bicarbonate. Curve 1 = phosphate increase after 0.2 hour (Experiment 10, pH 7.0); Curve 2 = phosphate increase after 0.25 hour (Experiment 12, pH 7.4); Curves 3 and 4 = total  $\text{CO}_2$  decrease (Experiments 12 and 10, respectively).

clearly that the amount of extra calcium (and  $\text{OH}$ ) in the precipitate is greater when the solution (at constant pH) contained a higher ratio of calcium to phosphate.

*Deductions Concerning Initial Formation of Precipitate*—The rate of  $\text{CO}_2$  uptake is similar to the rate of phosphate loss from the precipitate formed by adding Ca to an excess of phosphate. The rate of  $\text{CO}_2$  uptake and phosphate loss was found to be higher as the interval after the formation was decreased (Figs. 2 and 3). The uptake of  $\text{CO}_2$  by the precipitate was 40 to 75 per cent com-

plete in the first 5 minutes of equilibration (25 minutes after the addition of the calcium was begun). The rate of  $\text{CO}_2$  uptake was, therefore, highest during that time. Because the rate of phosphate loss was progressively decreasing from the time of the first analysis, it appears likely that its rate was also higher at an earlier time. Therefore, it would seem that the first aggregation of calcium and phosphate was  $\text{CaHPO}_4$ , which promptly lost phosphate to form the crystal lattice containing calcium and phosphate in the ratio of 3:2. If such is the case, the growth of the crystals would be expected to occur by a repetition of the process; namely, adsorption of the ions of  $\text{CaHPO}_4$  on the  $\text{Ca}_3(\text{PO}_4)_2$  with subsequent loss of phosphate to form additions to the crystal lattice. That process would be simply another case of adsorption by the  $\text{Ca}_3(\text{PO}_4)_2$  lattice of ions of calcium salts present in amounts near their solubility product. In other words, it would be essentially similar to the adsorption of  $\text{CaCO}_3$  and  $\text{Ca}(\text{OH})_2$ .

#### DISCUSSION

The above results indicate that the *initiation* of precipitation of the bone salt depends alone on the concentrations of calcium, phosphate, and hydrogen ions. The final composition of the bone salt is a function of the concentrations of ions, such as  $\text{Ca}^{++}$ , and  $\text{CO}_3^-$ ,  $\text{HPO}_4^-$ , or  $\text{OH}^-$ , which may be removed from solution by the precipitate originally formed. If the rate of change of composition of the bone salt during its formation approximates that observed by these precipitates in pure solution, it is to be expected that essentially the final composition would be attained after a few days. Precipitates (such as those in Experiments 130, 131, and 115) showed a detectable change in composition, however, even after equilibration for 20 days. The difference in proportion of  $\text{CO}_2$  to phosphorus of adult and young bone of certain species (13) may be related to this very slow change observed in the precipitates with long equilibration.

#### SUMMARY

1. Calcium phosphate precipitates formed at pH 7.0 to 7.4 in solutions containing bicarbonate remove  $\text{Ca}^{++}$  and  $\text{CO}_3^-$  from solution even though the product  $[\text{Ca}^{++}] \times [\text{CO}_3^-]$  is less than the solubility product of  $\text{CaCO}_3$ .

2. The change in the precipitates during equilibration (5 minutes or more after addition of the reagents) consists essentially of an increase of  $\text{CaCO}_3$  or  $\text{Ca}(\text{OH})_2$  content and loss of phosphate.

3. The composition of the precipitates after long equilibration depends on the composition of the liquid phase in respect to  $\text{Ca}^{++}$  and anions which may be adsorbed.

The results are considered in relation to the steps involved in the formation of the bone salts.

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## SOLUBILITY OF BONE SALT

### III. PARTIAL SOLUTION OF BONE AND CARBONATE-CONTAINING CALCIUM PHOSPHATE PRECIPITATES

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Previous evidence (1) has been given to indicate that during the formation of the bone salt the first crystalline substance formed removes ions of other calcium salts from solution and the amount removed increases as the product of these ions approaches the solubility product of the compound which they form. If this is so, it should be possible by changing the composition of the liquid phase to remove the adsorbed ions, or exchange them for others without dissolving the main crystal lattice.

Evidence is available to indicate that the composition of the solid changes when the composition of the liquid phase is altered. For instance, Klement (2) equilibrated the glycerol ash of bone with conductivity water and found that more  $\text{CO}_2$  than phosphate was lost from the solid. Likewise, Hendricks, Hill, Jacobs, and Jefferson (3) noted that more carbonate than phosphate is removed when bone is treated with steam.

It might be argued that in these experiments the bone salt dissolved and reprecipitated as a different and less soluble salt. Consequently, we have partially dissolved the bone salt and similar artificially prepared precipitates in such a way as to make reprecipitation highly unlikely, and it can be said at the start that most of the  $\text{CO}_2$  was removed apparently without dissolving the main crystal structure. Relative to the anions removed, the amount of Ca and other cations removed was greater when the precipitate contained a greater proportion of them. The results, therefore, are interpreted as additional indication that during the formation of the bone salt the addition of ions such as  $\text{CO}_3^{--}$



(with a cation) is a step which takes place subsequent to the formation of the main crystal structure.

The results also have a bearing on the practical question regarding the technique necessary in investigation of the composition of precipitates of this nature. Unaware of the extent to which the composition of such precipitates could be changed by washing, we at first attempted to follow the course of precipitation by analysis of both the liquid and solid phases. Precipitates examined immediately after their formation contained 15 to 20 per cent less carbonate than was expected from the composition of the liquid phase and the amount originally added. After establishing that the  $\text{CO}_2$  was lost from the precipitates during the process of washing them free from chloride, we adopted the procedure (described below) involving analysis of the moist unwashed precipitates and correction for the substances present in the adherent solution. This procedure completely accounted for the  $\text{CO}_2$  originally added as bicarbonate.

Preliminary experiments were carried out to find out to what extent the composition of precipitates similar to bone could be altered by washing with water under circumstances which would prevent reprecipitation. For this purpose, a continuous flow of  $\text{CO}_2$ -free distilled water was passed through precipitates (similar to that in Experiment 70-C) in an apparatus so constructed that the  $\text{CO}_2$  of the filtrate could be collected without loss. Following is a typical result: 5.81 gm. of a precipitate containing 0.826 mm of  $\text{CO}_2$  and 4.84 mm of phosphorus per gm. were washed with 10,890 cc. of water. The precipitate lost 32 per cent of the  $\text{CO}_2$  and 1.7 per cent of the phosphorus which it contained. The termination of the experiments resulted from clogging of the filter, whereby it became difficult to maintain a uniform flow through the precipitates.

In subsequent experiments, to avoid continuous filtration, solution of the solid was accomplished by continuous addition of dilute acid. To increase the rate of solution, the temperature was raised to  $100^\circ$ .

### *Procedure*

Two samples of the cortex of the femurs of cattle were cut into fine particles by means of a motor-driven, steel milling head. The

particles which passed a 30 mesh sieve, but which were held on a 40 mesh sieve, were heated with glycerol and KOH as described by Gabriel (4) to remove the organic matter. The resulting "glycerol ash" from 5 gm. of bone was washed with three successive portions of distilled water. A 2 gm. portion of each of the samples of glycerol ash was introduced into an apparatus, so arranged that water or acid could be added to the sample at 100° and the evolved CO<sub>2</sub> collected in towers containing known amounts of standard Ba(OH)<sub>2</sub>. The acid was added to the glycerol ash at a constant rate during the experiment and additional water was added so that the rate of dilution of the sample was constant. The pH of the solution after passing through the solid decreased below 7 by the time approximately half the acid and water was added, and at the end of the experiment was 5.5 to 6.0. When the volume of the solution added amounted to 3 to 4 liters, the solution was quickly filtered while hot, and analyzed for phosphate and calcium. The CO<sub>2</sub> absorbed by the Ba(OH)<sub>2</sub> was determined by titration as previously described (5). As a check on the accuracy of the procedure, the residue was dried at 100° and analyzed for phosphate and CO<sub>2</sub>.

Two samples of carbonate-containing calcium phosphate precipitates were investigated as described above for bone, except that the water and acid were added more rapidly. In these determinations, the addition of 3800 cc. of acid solution was completed in approximately 2 hours. The results are shown in Table I.

*Préparation of Precipitates*—Precipitate 106 was prepared as follows: To a flask calibrated at 2 liters, 49.0 mm of NaHCO<sub>3</sub> contained in 1200 cc. of water were added. To this were added, with rapid stirring, 90 mm of Na<sub>2</sub>HPO<sub>4</sub> and 120 mm of CaCl<sub>2</sub> and NaOH to keep the pH at 7.30. The phosphate and calcium chloride were added in thirty equal portions. The additions of the phosphate preceded the additions of the calcium, and the NaOH was added to bring the solution to the original pH after each addition of the calcium chloride. The solution was made to volume, the air space in the flask filled with molten paraffin, and the flask rotated at 38°. NaOH was added as required to keep the pH constant. After 3 days, the precipitate was centrifuged, and the adherent water removed as far as possible by pressure out of

TABLE I

*Substances Removed from Bone and Carbonate-Containing Calcium Phosphate Precipitates by Dilute Acid at 100°*

Experiment No.	Composition of glycerol ash of bone		Substances removed by acid from glycerol ash of bone								PO <sub>4</sub> of substances before acid treatment	PO <sub>4</sub> /CO <sub>2</sub> of residue	
	PO <sub>4</sub>	CO <sub>2</sub>	Ca	Mg	Na*	PO <sub>4</sub>	CO <sub>2</sub>	PO <sub>4</sub>	CO <sub>2</sub>	Calculated from substances removed		Determined by analysis	
	mm per gm.	mm per gm.	mm per gm.	mm per gm.	mm per gm.	mm per gm.	mm per gm.	per cent of total	per cent of total				
104	5.1	1.03	0.713	0.19	0.212	0.325	0.493	6.37	47.9	4.95	8.7	9.8	
105	5.12	1.03	1.10	0.185	0.197	0.515	0.594	9.9	57.6	4.97	10.6	10.5	
	Composition of ppt.		Substances removed by acid from ppts.										
106-A	5.58	0.723	0.21		0.25†	0.46	0.562	8.24	77.8	7.70	31.8	31.7	
70-C	4.84	0.83	0.17		0.25†	0.173	0.67	3.58	80.7	5.98	29.2	28.0	

*Amounts of Acid and Water Added per Gm. of Solid*

Experiment No.	Acid		Volume of water in which the acid was dissolved	Rate of addition
	mm			
104	1.3	Lactic	2100	9.76
105	1.05	Sulfuric	1600	8.90
106-A	0.9	"	1923	29.6
70-C	0.9	"	1880	32.7

\* The residues from the artificial precipitates gave essentially no test for base other than Ca. It is assumed that they originally contained 0.25 mm of Na per gm. The assumed value is that of the Na + K of bone and is 20 per cent higher than the Na content of similar precipitates washed just free of chlorides.

† The K removed was neglected for two reasons: (1) because, in relation to Na, bone contains only a small amount of K, and (2) KOH and glycerol were used in preparation of the glycerol ash.

contact with air, and transferred to a sealed vessel. Portions of the moist precipitate were withdrawn for water,  $\text{CO}_2$ , and phosphate analyses, and the solution was analyzed for  $\text{CO}_2$  and phosphate.

The amount of solution adhering to the precipitate was determined by drying a sample to constant weight at  $100^\circ$ . The  $\text{CO}_2$  and phosphate content of the precipitate was calculated by subtracting the  $\text{CO}_2$  and phosphate content of the solution adhering to the precipitate from the amounts found by analysis of the moist precipitate. The  $\text{CO}_2$  of the adherent water was less than 10 per cent of the total and the phosphate less than 3 per cent of the total.

Precipitate 70-C was prepared as follows: To 3 liters of solution containing 175 mm of  $\text{NaHCO}_3$  and 303.1 mm of  $\text{Na}_2\text{HPO}_4$ , were added 3 liters of solution containing 455 mm of  $\text{CaCl}_2$  and  $\text{NaOH}$  to keep the pH at 7.4. The reaction was carried out at  $37.5^\circ$ , and 15 minutes were required to complete the addition of the  $\text{CaCl}_2$ . The precipitate was agitated in contact with the solution for 20 days.

$\text{CO}_2$  was determined in solids with the aid of the Van Slyke and Neill manometric apparatus (6), equipped with a side tube, according to the procedure of Danielson and Hastings. Other analytical procedures were the same as previously employed.

### *Results*

The results given in Table I show that from the glycerol ash of bone, 47.9 and 57.6 per cent of the  $\text{CO}_2$  was removed, while 6.37 and 9.9 per cent respectively of the phosphate was dissolved. From the prepared precipitates, 77.8 and 80.7 per cent of the  $\text{CO}_2$  was removed, while 8.24 and 3.58 per cent respectively of the phosphate was dissolved.

It should be noticed that a difference exists between the sum of the bases and acids removed. From the glycerol ash of bone, the sums of the Ca, Mg, and Na removed were equivalent to 107 per cent (Experiment 104) and 101 per cent (Experiment 105) respectively of the sums of the  $\text{CO}_2$  and  $\text{PO}_4$  removed. From the precipitates, the sums of the Ca and Na removed were evidently equivalent to not more than 27 per cent (Experiment 106-A) and 32 per cent (Experiment 70-C) respectively of the sums of the  $\text{CO}_2$  and  $\text{PO}_4$  removed.

## DISCUSSION

The action of the acid on the glycerol ash of bone consisted essentially of removal of  $\text{CO}_2$  and phosphate with base (chiefly calcium) in amounts sufficient to indicate that essentially  $\text{CaCO}_3$  and  $\text{Ca}_3(\text{PO}_4)_2$  were removed from the bone. From the artificially prepared precipitates, the calcium removed was far from being equivalent to the anions.

The explanation for the difference apparently lies in differences in the relative  $[\text{Ca}^{++}]$ ,  $[\text{PO}_4^{=}]$ , and  $[\text{CO}_3^{=}]$  of the solutions from which the solids were formed. The artificial precipitates were formed from solutions containing excess phosphate. (This was done so that the product  $[\text{Ca}^{++}] \times [\text{CO}_3^{=}]$  would always be below the solubility product of  $\text{CaCO}_3$  during the precipitation.) The precipitates, therefore, had a composition represented essentially by the formula  $(\text{Ca}_3(\text{PO}_4)_2)_{10}(\text{CaHPO}_4)_{2.9}(\text{CaCO}_3)_{3.4}$  or a ratio of 1.43 moles of calcium combined per mole of phosphate.<sup>1</sup> Bone is formed from a solution of similar  $[\text{CO}_3^{=}]$  but the ratio of calcium to phosphate is presumably greater than that employed in the preparation of the artificial precipitates and, therefore, the composition of the bone is represented essentially by  $(\text{Ca}_3(\text{PO}_4)_2)_{10}(\text{CaCO}_3)_{4.5}$  (or 1.5 moles of calcium combined per mole of phosphate).<sup>1</sup> The artificial precipitates, therefore, contained a higher ratio of anions to cations. The undissolved residues remaining from the partial solution of both the bone and the artificial precipitates tend to approach the same composition; namely, a substance represented essentially by the formula  $(\text{Ca}_3(\text{PO}_4)_2)_{10}(\text{Ca}(\text{OH})_2)_{3.3}$  (7). Thus, the artificial precipitates lose relatively more of their anions than does the bone.

The exchange by the artificial precipitates of  $\text{CO}_2$  and  $\text{PO}_4$  for the  $\text{OH}$  evidently takes place in the solid phase, because the dilution and constant lowering of the pH in our experiments preclude the possibility that recrystallization could occur. This action, together with the removal of  $\text{Ca}$  and  $\text{CO}_3$  from the precipitate in the ratio corresponding to the formula  $\text{CaCO}_3$ , appears to afford an illustration of the manner in which the apparent  $\text{CaCO}_3$ ,  $\text{Ca}(\text{OH})_2$ , and  $\text{CaHPO}_4$  content of the precipitate may be altered during solution as during precipitation without disintegration of the main crystal structure.

<sup>1</sup> After subtraction of that combined with carbonate.

## SUMMARY

By means of continuous addition of dilute acid to the solid, more than half the  $\text{CaCO}_3$  can be removed from the glycerol ash of bone, while less than 10 per cent of the phosphate is removed. In the same way, most of the  $\text{CO}_2$  and only 4 to 8 per cent of the phosphate may be removed from calcium phosphate precipitates, which were prepared from solutions undersaturated with respect to  $\text{CaCO}_3$ . The relation of anions and cations removed under the conditions of the experiment is discussed in relation to the steps involved in the formation of the precipitates and bone.

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## MICROMETHODS FOR THE ESTIMATION OF DIASTASE

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Our studies of the diastatic activity of human blood and urine disclosed the fact that the existing micromethods devised for the purpose are inadequate for quantitative measurements. Curiously enough this applies to the fullest extent to methods which are based on the determination of the saccharogenic action of diastase, a procedure which involves a well elaborated, reliable analytical technique. The trouble in these methods consists in the lack of standardization of important factors such as hydrogen ion concentration and electrolyte content, which are known seriously to influence diastatic activity. Even the fundamental requirement of establishing proper relationships between enzyme activity and the amount of substrate is disregarded in some instances. Myers and Killian (1) for example employ with 2 cc. of plasma 10 mg. of starch, a quantity which is far from sufficient. As Sherman and his associates (2) have shown as early as 1910, a proportionality between enzyme activity and copper-reducing power prevails only when a very small fraction, no more than 5 to 6 per cent of the starch, is converted into reducing sugars (expressed as maltose). Myers observed with his method conversions up to 40 and 60 per cent, expressed as glucose, which in terms of maltose equals 60 to 90 per cent. These are excessively high values in view of the fact that 80 to 85 per cent is regarded as the upper limit of diastatic starch conversion (*Grenzextrin*). Under such conditions no quantitative relationship can exist between reducing power and enzyme activity, and the results obtained are erroneous. The inadequacy of 10 mg. of starch as substrate is even more obvious in abnormal cases (acute pancreatitis) in which 2 cc. of human blood plasma are capable of producing as much as 50 mg. of reducing sugars ("maltose") in 15 minutes.



An error frequently encountered consists in the extension of the reaction time to several hours, often to a day, and even longer. Workers resorting to such practise disregard the fact that the activity of diastase spontaneously deteriorates in solution, particularly at great dilutions. The rate of this loss of activity, we found, exhibits wide individual variations with different blood specimens, being nearly negligible in some instances, while mounting to 20 per cent or more in others in the course of a single hour. Hence results obtained in long reaction periods are misleading, even when used only in comparative studies.

In a second category of methods the initial phase of starch conversion, the amylolytic action of diastase, is observed as it is indicated by changing color reactions of the reaction mixture with iodine. These methods will be discussed later in this report.

The viscosimetric method is based upon the measurement of the decrease in viscosity of starch paste under the depolymerizing effect of diastase. This technique, as adapted for microdeterminations by Davison (3) and Elman and McCaughan (4), is attractively simple and rapid. We found, however, an inherent source of error in the procedure when used with blood plasma or serum; namely, due to a process of adsorption, mixed aggregates of protein and starch are formed which tend to separate out and to form a sediment. The phenomenon results in a diminution of viscosity as soon as plasma and serum are brought together. This change in viscosity is substantial and its independence of enzyme action can be demonstrated by employing serum which is devoid of diastatic activity. (Serum for this purpose may be inactivated by shaking it with starch powder and removing the starch by centrifugation. In two such operations starch completely adsorbs the diastase content of serum.) Owing to this defect the method does not yield quantitative values, and its inaccuracy is the greater, the lower the diastatic activity of the blood is. It is suitable, however, for the detection of abnormal elevations of blood diastase, as for example in cases of acute pancreatitis.

In view of the facts just outlined we devised two micromethods for the estimation of diastatic activity in blood, urine, and other biological material. The methods are quantitative in the sense that the quantities that are measured are in linear proportionality

with the diastatic activity. In the first method the saccharogenic action of the enzyme is determined with due regard to available information concerning the kinetics of the reaction<sup>1</sup> and to necessary precautions in the analytical technique involved (5). The elaboration of a second method, which is based upon the amylolytic action of diastase, was prompted by two objectives. In the first place, a technique, simpler and more rapid than the estimation of reducing power, seemed desirable in view of the clinical significance of diastase determinations. Secondly, we deemed it desirable to check and control the results of our copper reduction method by another method which is built on a basically different analytical procedure. Both purposes are served only if the measurement of the amylolytic action yields results which parallel those obtained in the determination of the saccharogenic action of the enzyme. Observations of Johnson (6) and of Sherman *et al.* point to the existence, under certain conditions, of such parallelism; our task was to establish these conditions for a micro-technique.

#### *Method Based on the Estimation of Saccharogenic Activity*

Sherman, Kendall, and Clark (2) devised in 1910 a method of great perfection for the estimation of diastatic activity on the basis of the saccharogenic action of the enzyme. The procedure is readily adaptable to serve as a micromethod which permits the reliable estimation of the diastatic activity of blood serum. This activity is in general very slight in human blood, 1 cc. of serum producing in extreme instances no more reducing matter in 30 minutes than corresponds to the copper-reducing power of 0.1 mg. of glucose.

We adopted from Sherman's method the reaction period of 30 minutes and the temperature of 40° as standard conditions. The selection of substrate concentration and other experimental conditions was based upon our preceding studies of the kinetics of the enzymatic reaction,<sup>1</sup> specifically with blood diastase, which yielded results that are in good accord with the findings of Sherman and his associates. Guided by this information we combined conditions under which the copper-reducing power imparted to starch by diastase shows direct linear proportionality to enzyme

<sup>1</sup> Experiments of the author, soon to be published. .

activity; *i.e.*, under which doubling and trebling of the amount of diastase leads to doubling and trebling, respectively, of the copper-reducing power of the reaction products.

### *Reagents*

*Starch Paste*—We employ U.S.P. corn-starch or pure rice starch. The starch is washed as follows: 100 gm. of starch are suspended and frequently agitated for about an hour in 1 liter of approximately 0.01 N HCl. After sedimentation the acid is poured off and the starch is stirred up in 1 liter of approximately 0.05 per cent NaCl solution. After sedimentation and decantation washing with salt solution is repeated once more; then the starch is spread out and allowed to dry in air.

A paste is prepared with approximately 15 gm. of washed starch per liter of water. The starch is thoroughly ground in a mortar with 50 cc. of water, while 900 cc. of water are heated to boiling. The ground starch suspension is transferred into the hot water with vigorous agitation, 50 cc. of water being used to rinse the mortar. After boiling for 0.5 to 1 minute (with agitation), the starch paste is heated in a water bath for 15 to 30 minutes. The mouth of the flask is kept covered by an inverted beaker during the heating period. Grinding exerts the favorable effect of keeping the starch in a well dispersed state for a long time, whereas without the grinding it soon forms a heavy sediment.

*Acid NaCl Solution*—This contains 10 gm. of NaCl and 3 cc. of 0.1 N HCl per liter. The presence of the acid is necessary for correction of the pH of blood serum or plasma which tends to rise above the upper limit of the optimum pH range (7.0 to 7.4) owing to loss of CO<sub>2</sub> during the preparatory manipulations.

*Protein Precipitants*—A 5 per cent solution of CuSO<sub>4</sub>·5H<sub>2</sub>O and a 6 per cent solution of sodium tungstate are used for the deproteinization of plasma or serum. For whole blood 7 per cent copper sulfate and 10 per cent sodium tungstate are employed (7).

*Copper Reagent of High Alkalinity*—This reagent, used for the determination of reducing sugars, is similar to the one described in a previous paper and is prepared according to the directions given there (8), with the one difference that Na<sub>2</sub>SO<sub>4</sub> is left out from among its constituents. The reason for this omission is

that the filtrates to be analyzed in this procedure contain appreciable quantities of undigested starch which, in the course of the sugar determination, coagulates in the presence of  $\text{Na}_2\text{SO}_4$  and interferes with the iodometric determination of the reduced copper. For convenience we present in Table I the glucose equivalents of reduction values obtained with this reagent.

TABLE I

*Glucose per 100 Cc. of Solution Corresponding to Titration Values When 5 Cc. of 1:10 Solution and 5 Cc. of High Alkalinity Copper Reagent Are Heated in Water Bath for 20 Minutes*

0.005 N thio- sulfate	0.005 N sodium or thiosulfate									
	0	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.7 cc.	0.8 cc.	0.9 cc.
	Glucose in 100 cc. of blood or plasma									
cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0					18	22	26	29	32	35
1	39	42	45	48	52	55	58	61	65	68
2	71	74	78	81	84	87	90	93	96	99
3	102	106	109	112	115	118	121	124	127	130
4	133	137	140	143	146	149	152	155	158	161
5	164	168	171	174	177	180	183	186	189	192
6	195	199	202	205	208	211	214	217	220	223
7	226	229	232	235	238	241	244	247	250	253
8	256	260	263	266	269	272	275	278	281	284
9	287	290	293	296	299	302	305	308	311	314
10	317	321	324	327	330	333	336	339	342	345
11	348	352	355	358	361	364	367	370	373	376
12	379	383	386	389	392	395	398	401	404	407
13	410	414	417	420	423	426	429	432	435	438
14	441	445	448	451	454	457	460	463	466	469
15	472	475	478	481	484	487	490	493	496	499
16	502	506	509	512	515	518				

### *Analytical Procedure*

In a test-tube of 14 to 16 mm. diameter 5 cc. of the starch paste and 2 cc. of the  $\text{NaCl}$  solution are mixed and immersed in a water bath of  $40^\circ$ . After a few minutes, when the fluid has assumed the temperature of the water bath, 1 cc. of plasma (serum, whole blood) is added, the tube is stoppered and inverted a few times, and the mixture is incubated for 30 minutes. At this point the

action of the enzyme is terminated in conjunction with the deproteinization of the reaction mixture. To this end 1 cc. of the  $\text{CuSO}_4$  solution is admixed, then 1 cc. of the tungstate solution is added, and the mixture is well shaken and centrifuged. Since copper salts inhibit but do not fully stop diastase action, these operations must be carried out without undue delay.

The supernatant fluid, which is enzyme-free, is poured through a small filter paper. The filtrate, a 1:10 dilution of the plasma, is opalescent owing to the presence of unaltered starch, and is ready for the determination of its copper-reducing power. This is done by heating 5 cc. of filtrate with 5 cc. of the "high alkalinity" copper reagent for 20 minutes. For the rest the procedure follows the Shaffer-Hartmann technique (9).

On acidification the liberated iodine gives a strong blue color with the excess starch that has been carried over into the filtrate. This dark color makes it difficult to observe the point where the white cuprous iodide has gone completely into solution. To avoid errors on this account it is well to agitate the fluid frequently and long enough before titration with thiosulfate, in order to enhance oxidation and dissolution of the iodide. Again, at the titration special care must be exercised shortly before the end-point is reached, since the undigested starch present, which is considerable in amount, in particular when the diastatic activity had been low, tends to hold the last remnant of free iodine so firmly adsorbed that the blue starch-iodine complex may persist for a brief period in the presence of excess thiosulfate, and overtitration may be the consequence. In order to avoid such error, it is advisable to stopper and shake the contents of the test-tube shortly before the titration is completed.

The glucose equivalent of the titration figure, obtained from Table I, represents reducing substances corresponding to 100 cc. of plasma (serum, whole blood) and comprises the glucose originally present plus the reducing matter formed by the diastase content of 100 cc. of plasma. The plasma glucose is determined in a separate analysis as follows: 1 cc. of plasma is diluted with 7 cc. of water and deproteinized with 1 cc. of 5 per cent copper sulfate and 1 cc. of 6 per cent tungstate. In the filtrate glucose is determined with the high alkalinity reagent. The glucose equivalent is read from Table I.

*Diastatic Activity (Definition)*

After the glucose content of the plasma is deducted from the total reducing substances, the difference represents the reducing power (in terms of glucose) of the reaction products formed from starch by the diastase content of 100 cc. of plasma. This figure we denote as diastatic activity. Thus, when we say that the diastase value of a sample of blood plasma is 120, we mean that, under the standardized conditions described, 100 cc. of that plasma produce from starch cleavage products which have the same copper-reducing power as 120 mg. of glucose.

The diastatic activity of the blood plasma of healthy individuals ranges from 80 to 150, with occasional extensions of moderate degrees beyond either limit. In many pathologic conditions considerable deviation from the normal values in both directions can be observed. Our method has been particularly useful in the accurate estimation of subnormal values, which may drop as low as 20 and even lower. Values above normal can be reliably determined only up to a limit of 400. Diastatic activity above this limit causes the conversion of more than 5 per cent of the substrate into reducing matter (in terms of glucose) and as a consequence impairs the proportionality between the amount of diastase and the reducing matter produced. In such cases dilution of the plasma with 0.5 per cent NaCl solution overcomes the difficulty. 1 cc. of the diluted plasma is used, and the result is multiplied by the dilution factor. The extent of dilution depends, of course, on the height of diastatic activity. The highest diastatic activity we observed in human blood plasma was over 3000, so that at least a 1:7 dilution was necessary. In most instances of elevated diastatic activity, however, dilutions of 1:2 or 1:3 are satisfactory.

*Method Based upon Color Reaction with Iodine*

The initial step in the elaboration of this procedure consisted of a detailed scrutiny and standardization of all the factors which affect the reaction, foremost among them the selection of the end-point and of the object of measurement.

*Selection of End-Point*—In selecting the end-point we found that the achromatic point (that stage of the reaction at which

the reaction mixture ceases to give color with iodine) must be left out of consideration in a micromethod in which the enzyme solution is represented by a small amount of blood plasma, often of very slight diastatic activity. The achromatic point under such conditions is reached only after protracted incubation, and occasionally not at all, since complete spontaneous inactivation of the enzyme may precede the depolymerization of the last of the starch to the stage of achroodextrins. For this reason we adopted as the end-point that stage of the reaction at which the violet hue just disappears, yielding to the brownish red color characteristic of erythrodextrins. Before this stage is attained, the transition from blue to red proceeds over a continuous series of purple shades, without any line of demarcation. Then, after the disappearance of the last remnant of purple, the erythro-dextrin-iodine color fades into more and more pale brown and yellow shades as the enzyme reaction progresses, until the achromatic point is reached. This transition, too, is quite gradual. The only sharp turning point, suitable for adoption as an end-point, is the stage at which the last tinge of purple disappears.

We increased the accuracy in the observation of the end-point by preparing a clear, transparent starch solution which does not become turbid when mixed with blood plasma and subsequently with iodine solution. With this starch solution minute changes in the color reaction can be discerned when viewed in intensive transmitted light. A 100 watt frosted electric light bulb serves as the standard source of light, providing uniform intensity of illumination, independent of changes in daylight.

The quantity of iodine employed in the color reaction requires careful standardization, since the yellow, which is introduced with an excess of iodine, as a complementary color neutralizes purple and, as a consequence, distorts the end-point. Lack of regard to this factor may entirely vitiate the results of a micromethod.

*Object of Measurement*—In most of the known methods the reaction time and the amount of the substrate are fixed as standards, the object of measurement being the amount of the enzyme preparation that is required to carry the reaction to the end-point. In this technique, graduated quantities of the enzyme solution are introduced into a series of receptacles, each charged with a standard quantity of starch, and the reaction is allowed to

proceed at 40° for 30 minutes. At the end of this time the receptacle is sought out in which no purple or no color at all is given with iodine, depending on the choice of the end-point. This procedure is tedious and relatively crude as applied, for example, in Wohlgemuth's (10) method, widely used for the assay of blood diastase. In this method, when the amount of plasma is serially diminished by as little as 0.1 cc. in each successive test-tube, there is a difference of 25 per cent in the enzyme content of the tubes with 0.4 and 0.5 cc. of plasma, and a gap of 100 per cent between the two reaction mixtures which contain 0.1 and 0.2 cc. of plasma. In cases where the end-point had not been reached in one tube and had been passed in the next, interpolation with acceptable accuracy is scarcely possible. Thus, in order to obtain more accurate results, one would have to follow the directions of Johnson (6) who in such instances prescribed repetition of the entire procedure with enzyme solutions diluted on the basis of the information gained from the first assay.

A more accurate and far more simple technique can be elaborated, we found, by adopting time as the object of measurement. A single reaction mixture of standard starch content and of standard total volume is prepared from which periodically samples are withdrawn for testing with iodine until the end-point has been reached. The time required is recorded.

*Other Factors*—In regard to pH and electrolyte concentration we found that the optimum conditions are the same in the amylolytic process as in the saccharogenic reaction. We also have retained 40° as the standard temperature.

*Proportionality*—With application of the foregoing standardizations, *i.e.* fixed amount of starch in a fixed volume of the reaction mixture and a well defined end-point, conditions could be established under which linear proportionality obtains between the amount of diastase and the time required to carry the reaction to the end-point. It may be stated without going into details that this requirement is satisfied when the quantitative relationship between substrate and enzyme is such that the reaction reaches the end-point within 8 to 20 minutes.

When adapting these conditions to a microtechnique for the assay of blood plasma, no more than 1.0 cc. of plasma should be required. The adequate substrate for this is 3.0 mg. of starch,



an amount which is digested to the end-point by 1 cc. of normal human blood plasma within 8 to 20 minutes. We settled on this range as practical, since the maximum (20 minutes) is not unduly long, and the minimum (8 minutes) is long enough to allow a sufficiently accurate recording of time. The inverse proportionality between diastatic activity and reaction time in this combination of conditions is readily demonstrable: if the reaction time with 1 cc. of plasma is 10 minutes, 0.5 cc. of it requires 20, and 0.33 cc. 30 minutes.

Accordingly, the amyloclastic activity of blood plasma is represented by the expression,  $1/(t \times v)$ , in which  $t$  = reaction time in minutes and  $v$  = volume of plasma in cc. Provided that the amyloclastic action of plasma, as determined by this technique, runs parallel with the diastatic activity,  $D$ , as determined by the copper reduction method, then  $D = K \times 1/(t \times v)$ ,  $K$  being a constant that can be calculated if both  $D$  and  $t$  are determined on a series of plasma specimens. Figures accumulated on hundreds of samples show  $K$  actually to represent a constant of an approximate value of 1600, with some deviations due in the main to the variability in the extent of deterioration of the enzyme during incubation.

### *Reagents*

*Starch Solution*—This reagent contains 75 mg. of starch and 250 mg. of NaCl per 100 cc. of solution. It is prepared of the same washed corn-starch as is used in the copper reduction method. In preparing a paste as the first step, 15 to 20 gm. of the starch are rubbed up in a mortar with 100 cc. of water and the suspension is poured with vigorous agitation into 900 cc. of boiling water. After this has boiled for about 1 minute under continuous stirring, 10 cc. of 25 per cent NaCl solution are added and the flask is immersed for 30 minutes in a boiling water bath, the mouth of the flask being covered with an inverted beaker. After standing (covered with the beaker to keep the solution sterile) at room temperature for a day (or longer if necessary), the greater part of the starch separates out, forming a sediment. The limpid supernatant fluid is removed by syphoning or, in order to obtain a better yield, by centrifugation. This dilute starch paste is the stock

solution which, properly diluted with 0.25 per cent NaCl solution, furnishes the substrate.

To find the extent of dilution, the starch content (usually 0.4 to 0.6 per cent) of the stock solution is determined as follows: Into a large test-tube ( $25 \times 200$  mm.) 5 cc. of the fluid are introduced, 1 cc. of 3.6 N HCl is added, the tube is closed with a 1-hole rubber stopper that is fitted with a glass tube about 2 feet long, to serve as a reflux condenser, and the mixture is immersed in a boiling water bath and heated for 2.5 hours. The hydrolysate is neutralized with NaOH (phenol red indicator), diluted to 100 cc., and the glucose content is determined. This, multiplied by 0.9, represents the starch content of the stock solution and serves as the basis for dilution. For example, if the concentration of starch was found to be 0.428 per cent, 75 cc. of the solution are measured into a 500 cc. graduated cylinder and diluted with 0.25 per cent NaCl solution to 428 cc. The diluted solution then contains 75 mg. of starch and 250 mg. of NaCl per 100 cc.

The reagent must be kept sterile, since the slightest contamination with microorganisms destroys its usefulness. At the same time, however, it must be accessible to daily use. We satisfy both requirements by putting up the reagent in a flask which is closed with a 2-hole rubber stopper, one hole holding a syphon tube, and the other an air inlet tube. The latter is filled with sterile cotton. When putting up the syphon flask, we sterilize by heating the contents to boiling over an open flame, then continue the heating for 2 hours in a water bath. Sterilization in this manner is repeated twice more during the next 2 or 3 weeks. A reagent thus prepared keeps indefinitely for practical purposes. The syphon tube is kept filled with the reagent and is closed with a pinch-cock. A small rubber bulb filled with alcohol (such as is used for medicine droppers) is slipped on the glass tip of the syphon when not in use. Before the reagent is used, a few drops are run out from the syphon to rinse away the adhering alcohol.

In this laboratory we usually put up 6 liters of the starch reagent at a time, which suffice for over 1000 diastase determinations. The stock reagent, of course, also may be kept indefinitely if properly sterilized.

*Iodine Solution*—This is a 0.002 N aqueous iodine solution con-

taining 2 per cent of KI. It is prepared by dilution of 10 cc. of a 0.1 N aqueous iodine solution to 500 cc. with a 2 per cent KI solution.

### *Analytical Procedure*

Into a test-tube of 14 to 16 mm. diameter, 4 cc. of the starch solution (which contains 3.0 mg. of starch) are introduced, and the tube is immersed in the constant temperature water bath. A few minutes later, when the reagent has assumed the standard temperature, 1 cc. of the blood plasma (serum) under examination is added, and simultaneously a stop-watch is started. The mixing of the plasma and starch is expedited by blowing the plasma from a 1 cc. pipette ("to contain") into the reagent and rinsing the pipette once with the mixture.

While the reaction mixture is being incubated, 0.5 cc. portions of the iodine solution are measured into test-tubes. These test-tubes are of about 7 mm. inside diameter and should be fairly uniform. After about 5 to 6 minutes of incubation a 0.5 cc. portion of the reaction mixture is withdrawn with a pipette and added to one of the iodine tubes for observation of the reaction. The test-tube is viewed in transmitted light, coming from a standard source. This consists of a 100 watt frosted electric light bulb covered by a cardboard screen with a slit in the front. At subsequent time intervals further 0.5 cc. samples are withdrawn from the reaction mixture for color tests with iodine, until the red-brown color of erythrodextrin is seen with barely a perceptible tint of purple. At this point, the end-point, the duration of the reaction is registered on the stop-watch.

In practise this technique is exceedingly simple. After a few trials one is able to judge with fair approximation the particular shade of purple which appears when the reaction had progressed approximately half way. If this stage is attained in about 5 minutes (*i.e.*, at the time when the first sample is withdrawn and tested), the subsequent samples are best withdrawn at 2 minute intervals until the red-brown shade becomes predominant over the purple. From this point on, samples are tested at 1 minute intervals until the end-point is reached. Should the reaction have proceeded beyond half way in 5 or 6 minutes, samples are tested every minute thereafter. Again, if after 5 minutes the

first color test shows more blue than purple, indicating that the cleavage of the starch has just started, one allows a greater interval of time to elapse before the next test is performed. Experience soon teaches one to decide on the length of time that can be permitted to elapse between two color tests, so that after some practise the analysis can be carried out with only 2 cc. of starch solution and 0.5 cc. of plasma. As a matter of fact, these are the quantities we have been using for several years.

When the diastase content of the blood is abnormally high, as for example in acute pancreatitis, the color given with iodine will be far past the end-point at the 5 minute test. In such instances the determination is repeated with diluted plasma. The extent of the dilution is indicated by the shade of color obtained in the first tests with iodine. Dilutions of 1:2 to 1:15 may be necessary. For dilution of the plasma 0.5 per cent NaCl solution is used.

In a number of pathological conditions, notably in those resulting in impaired liver function, the blood diastase is lower than in the healthy subject. In such cases the reaction time extends beyond 30 minutes, not infrequently up to 60 minutes and even longer. As stated previously, the results are correct only when the reaction reaches the end-point within 30 minutes; but extension of the reaction time to 40 or 60 minutes still is compatible with the degree of accuracy required for most purposes. Further prolongation of the reaction time, however, due to the progressive inactivation of the enzyme, leads to results that are somewhat below the accurate values. This source of inaccuracy can be eliminated in these exceptional cases by using for substrate 1.5 instead of 3.0 mg. of starch. Since the standard volume of the reaction mixture must be maintained, the substrate containing 1.5 mg. of starch is prepared by diluting 2 cc. of the standard reagent with 2 cc. of 0.25 per cent NaCl solution. Decreasing the starch to half of the standard amount cuts the reaction time in half, so long as the end-point is reached within 30 minutes.

It is not superfluous, perhaps, to reiterate at this juncture the importance of using the correct amount of iodine. The standard quantity of iodine recommended as a rule satisfies two requirements: part of it is consumed by unsaturated constituents of the plasma, and the rest is just sufficient to combine with the starch

without leaving an excess that would distort the end-point by complementing purple shades. The relatively high KI content of the iodine reagent serves to stabilize the colors. Without this addition the iodine is consumed by organic matter in the mixture so rapidly that in a few minutes none is left in combination with starch, and the colors fade out. This may happen also with our standard iodine reagent, but only on rare occasions when the plasma contains unusually large amounts of unsaturated substances (lipids). The trouble is remedied by adding dropwise 0.002 N iodine until maximum color intensity appears. Conversely, when the plasma is diluted more than 2-fold in cases of abnormally high diastatic activity, the standard 0.5 cc. of iodine solution may become excessive, and only 0.3 cc. is to be used. If these precautions are observed, the resultant colors are clear and brilliant in transmitted light, and permit one to discern very slight changes in hue.

It is evident that subjective factors play a considerable part in this technique as in all methods based on the judgment of color changes in the starch-iodine reaction. We endeavor to restrict the influence of the personal element by standardization of every step; yet we consider it imperative that anybody who wishes to use this method check the results by the copper reduction method until the attainment of security in the technique.

*Calculation of Results*—From the equation  $D = K \times 1/(t \times v)$ , the diastatic activity can be obtained once  $K$ , the constant factor, has been experimentally determined. As stated before, under the standard conditions described,  $K = 1600$ . Thus, for example, when 12 minutes are required for 1 cc. of plasma to digest 3 mg. of starch to the selected end-point of the reaction,  $D = 1600/12 = 133$ ; or, when the reaction time for 1 cc. of a 1:4 dilution of plasma (actually 0.25 cc. of plasma) is 13 minutes,  $D = 1600/(13 \times 0.25) = 492$ . In case 1.5 mg. of starch are employed for the assay of very low diastatic activity,  $K = 800$ , provided that the reaction time does not exceed 30 minutes.

The values obtained in this manner are in close agreement with those obtained by the copper reduction method. There is no need, therefore, to introduce any specific "diastase unit," to express diastatic activity as determined on the basis of the amylolytic activity of the enzyme.

The numerical value of  $K$  depends on several factors. The foremost among these is the quality of the substrate. Different samples of the starch reagent, prepared in this laboratory from various batches of corn or rice starch in the course of several years, showed no substantial variations in the value of  $K$ . Older samples, however, that stood in the laboratory for several years after being washed, yielded somewhat turbid solutions, and a value of 1800 to 2000 for  $K$ . It is noteworthy that potato starch cannot be used in this procedure because it gives with iodine indefinite, pale color shades.

Another factor, the subjective element that enters into the appraisal of color shades, may appreciably affect the reading of the end-point; *i.e.*, the time factor. The use as the standard source of light of some device that differs from ours (as for example the use of a blue glass light bulb) is still another factor which affects the reading of the end-point. All these variables are absorbed in the value of  $K$  as its components, and as a consequence they can be ruled out as possible sources of error by determining  $K$  under any particular conditions selected as standards. Thus, instead of considering the technique as described above as a stereotyped formula, every competent worker may adapt it to special needs by selecting standard starch concentrations (one may increase it up to 0.4 per cent) and volumes which differ from those just described. The two main limiting factors are the requirements that linear proportionality must obtain between enzyme concentration and reaction time, and that the reaction mixture must be fairly transparent.

Simplicity and rapidity of the procedure are appreciable assets in clinical work, as also in obtaining quick orientation as to the range of the diastatic activity of an unknown material. Another advantage of the method is that, with very dilute starch solutions, it permits the detection of minute amounts of diastase which escape detection with any copper reduction method. This holds particularly for cases in which diastase is to be identified in solutions which contain considerable amounts of copper-reducing matter.

A limitation of the method is that it can be used only with colorless or slightly colored enzyme solutions which are transparent or at least translucent. It is unfit, for example, for the assay of

whole blood, of strongly lypemic or excessively icteric plasma, etc. In such instances one must resort to the copper reduction method.

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## CYSTINURIA

### VIII. THE METABOLISM OF CRYSTALLINE EGG ALBUMIN

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Previous investigations in cystinuria have shown that methionine, cysteine, and homocysteine yield extra cystine in the urine, while cystine, homocystine, and glutathione are oxidized to inorganic sulfate (1, 2). Methionine and cystine, fed as constituents of casein and lactalbumin (3), are catabolized both qualitatively and quantitatively in the same way as when they are administered in the form of the free amino acids. Since casein and lactalbumin are not homogeneous proteins, it was thought desirable to study the catabolism of crystalline egg albumin in cystinuria.

*Case History and Diet*—The cystinuric patient A (*cf.* (3)) has been maintained at home on a weighed diet which contains somewhat more protein and sulfur than previously. He rigidly adheres to his diet, as can be seen from the nitrogen and sulfur figures for the control periods (Table II, Periods A48, A50, A52, and A54). Up to the present (January, 1938), there has been no evidence of new stone formation, as determined by frequent x-ray observations.

*Analysis of Crystalline Egg Albumin*—We are indebted to Dr. H. O. Calvery for a preparation of crystalline egg albumin, designated as Batch C; this was recrystallized once and denatured (*cf.* (4)). No special precautions were taken during the denaturation and subsequent drying to prevent oxidation of sulfhydryl groups (5), so that the cysteine content of Batch C is very low.



The analysis of Batch C was given in a previous paper (6) and is summarized in Table I. Inorganic sulfate S, cystine S, cysteine S, and methionine S account for 98 per cent of the total S of the protein.<sup>1</sup> The N:S ratio of Batch C is 9, while the ratios N to methionine S and N to cystine plus cysteine S are 13 and 33 respectively.

*Methods*—The values in Table II were determined by the methods discussed in former publications (1, 2, 8). The Sullivan method was carried out as described previously ((9), cf. (10)) and also by Sullivan and Hess' recent modification (11), designated in Table II

TABLE I  
*Analysis of Denatured Crystalline Egg Albumin, Batch C*

	per cent	per cent as S	per cent of protein S
Nitrogen.....	14.9		
Total S*.....	1.81		
Inorganic sulfate S.....	0.20		
Protein S (by difference).....	1.61		
Methionine.....	5.2	1.13	70
Cystine†.....	1.38	0.38	24
Cysteine†.....	0.25	0.07	4
			<i>ratio</i>
N to protein S.....			9
" " methionine S.....			13
" " cystine + cysteine S.....			33
Methionine S to cystine + cysteine S.....			2.5

\* Pregl-Saschek method (7). Determinations by the Benedict method give low results on account of the high methionine content.

† Determined photometrically (8).

as the Sullivan B method. With the Sullivan B procedure, the effect of interfering substances (2, 9, 12) is decreased, but not entirely eliminated, since the values for cystine during control periods were still about 5 per cent lower than by the photometric method (8). During experimental periods the discrepancy between the two methods was raised to about 10 per cent, apparently due to the increased excretion of substances which interfered with

<sup>1</sup> Since this paper was submitted for publication, we have obtained evidence that cysteinesulfinic acid accounts for about 3 per cent of the protein S denatured egg albumin.

color development in the Sullivan B procedure. This conclusion, *viz.* that the photometric method gives correct results, seems justified, because in repeated experiments with a number of cystinuric patients agreement was obtained between the photometric and Lugg-Sullivan<sup>2</sup> methods both in control and experimental periods. For the calculations in Tables III to V, the cystine values obtained by the photometric method were used.

*Metabolic Observations*—The excretion of the various urinary constituents (see Table II) was constant during the control periods. Throughout the experiment there was little change in the volume of the urine. During the experimental periods, there was a slight rise in the excretion of ammonia and of undetermined nitrogen. These values were omitted from Table II. Determinations for homocystine carried out by a modification of the Folin photometric method (2, 8, 13) were always negative. Tests for sulfhydryl compounds in the urine were negative throughout the experiment.

*Creatinine Excretion*—Both during control and experimental periods there were only minor variations in the excretion of pre-formed creatinine, indicating conditions satisfactory for metabolic experimentation. Ingestion of 100 gm. of crystalline egg albumin did not result in a rise in the creatinine output.

*Methionine (Periods A49 and A49a)*—Lewis, Brown, and White (14) have recently found that, when their cystinuric patient received a high protein diet, the extra cystine excretion after the administration of methionine was less than that observed under similar conditions when a diet of lower protein content was fed. In our earlier series of experiments (1) the total N, total S, and cystine excretion of patient A were about 6.0, 0.56, and 0.76 gm. per day, respectively, during control periods ((1) Table I, Periods 9, 11, 13, 15, etc.), while in the present series the daily excretion of total N, total S, and cystine was about 8.0, 0.70, and 1.0 gm., respectively. It was therefore desirable to establish the sulfur partition after the administration of methionine at the higher level of protein intake, since we intended to use the data for the evaluation of the egg albumin experiment.

<sup>2</sup> For the determination of cystine in urine by the Sullivan method, we prefer (in spite of the somewhat low results) the Sullivan B procedure to the more troublesome Lugg-Sullivan method.



In Period A49 (*cf.* Table II) the same amount of methionine (8 gm.) was fed as in Period 14 ((1) Table I). A summary and an analysis of the data are given in Tables III and IV (the method of calculation was described in detail in previous publications (1, 3)). It can be seen that about 15 per cent less cystine and about 15 per cent more inorganic sulfate were excreted at the higher protein level of the present experiment.<sup>3</sup>

The high undetermined neutral S in the methionine experiments is apparently not due to the excretion of an S—S compound other than cystine, since negative results were obtained with the modified Folin photometric method (*cf.* (2, 8, 13)). (This method clearly showed the presence of homocystine in the urine after feed-

TABLE III  
*Extra Nitrogen and Sulfur in Experimental Periods*

	<i>dl</i> -Methionine, Periods A49, A49a			Egg Albumin C, Periods A53, A53a		
	Fed	Excreted		Fed	Excreted	
	gm.	gm.	per cent	gm.	gm.	per cent
Amount.....	8.0			100.0		
Extra N.....	0.75	(-3.0)*		14.9	7.5	50
“ total S.....	1.72	1.07	62	1.81	1.09	
“ SO <sub>4</sub> -S.....				0.20	0.20†	100†
“ protein S.....				1.61	0.89	55

\* Nitrogen-sparing action.

† It is assumed that the inorganic sulfate was completely excreted.

ing homocystine and homocysteine (2).) The fact that in all our experiments *dl*-methionine was fed may account for the high excretion of undetermined neutral S. This will be checked in the future with the *d* and *l* isomers.

In agreement with former experiments, a nitrogen-sparing action of methionine was observed (*cf.* Table III).

\* We did not have the opportunity to carry out a cysteine experiment on the present diet. Lewis has shown that the catabolism of cysteine varies with the protein intake in the same way as that of methionine. The partition of the extra S after cysteine feeding found in the earlier experiment ((1) Table I) was therefore recalculated for the present protein intake; on this basis cysteine would yield 55 per cent of the extra S as cystine and 45 per cent as inorganic sulfate.

*Crystalline Egg Albumin (Periods A53 and A53a)*—The ingestion of 100 gm. of Batch C resulted in a considerable increase in the excretion of cystine and of inorganic sulfate (*cf.* Table II). The excretion of the extra S and of extra cystine continued for several days in the adjustment period.

The metabolic data are summarized in Tables III and IV. The excretion of extra N and extra protein S was 50 and 55 per cent, respectively, of the amounts fed, so that the N to protein S ratio of the fed, catabolized, and "stored" protein was about the same (*i.e.* 9). It is possible that the uniformity of the N:S ratio would be altered considerably if the feeding experiment was repeated under different dietary conditions, but on the other hand, this

TABLE IV  
*Extra Cystine and Partition of Extra Sulfur in Experimental Periods*

Substance	Period No.	Extra S excreted as						Extra cystine excreted
		Total S	Inorganic SO <sub>4</sub> -S		Undetermined neutral S		Cystine S	
		gm.	gm.	per cent	gm.	per cent	gm.	per cent
<i>dl</i> -Methionine*	A14, A14a	1.10	0.37	33	0.17	15	0.55	50
"	A49, A49a	1.07	0.43	40	0.19	18	0.45	42
Egg Albumin C	A53, A53a	0.89†	0.51†	57	0.08	9	0.30	34

\* *Cf.* (1).

† Corrected for 0.20 gm. of inorganic sulfate S fed (*cf.* Table III).

uniformity may have some relationship to the catabolism of egg albumin. Clearly, further experiments are needed to decide this question.

In the experiment with Batch C, the extra cystine (1.12 gm.) accounted for 34 per cent and the inorganic sulfate for 57 per cent of the extra protein S (Table IV). These data and the analogous data after the feeding of casein and lactalbumin ((3) Table V) make it evident that the excretion of extra cystine and the partition of the extra S are dependent on the distribution of the S between cystine and methionine in these three proteins. This point can be more conclusively demonstrated by calculating the partition of the extra S as described previously in detail for the casein and lactalbumin experiments ((3) Tables VI and VII).

The partition of the extra S in the egg albumin experiment is calculated in Table V and is in agreement with that actually found.<sup>4</sup>

It appears that, under the conditions of this experiment, cystine and methionine (and cysteine) fed as denatured crystalline egg albumin were catabolized both qualitatively and quantitatively in the same way as when they were administered in the form of the free amino acids. No appreciable reduction of cystine to cysteine seems to have occurred, since all of the extra cystine excreted was

TABLE V  
*Egg Albumin (Periods A53 and A53a). Partition of Extra Sulfur,  
Calculated and Found*

The values are given in gm.

Partition of extra S calculated from catabolism of	Inorganic SO <sub>4</sub> -S	Undeter- mined neutral S	Cystine S	Cystine
0.20 gm. sulfate S +	0.20	0.00	0.00	0.00
0.21 " cystine " +	0.20	0.01	0.00	0.00
0.04 " cysteine "† +	0.02	0.00	0.02	0.08
0.62 " methionine S‡	0.25	0.11	0.26	0.98
Calculated	0.67	0.12	0.28	1.06
Found (cf. Table IV)	0.71	0.08	0.30	1.12

\* Calculated on the basis that cystine is catabolized 94 per cent to inorganic sulfate and 6 per cent to undetermined neutral S ((1) and unpublished experiments at the present level of N intake).

† Calculated on the basis that cysteine is catabolized 45 per cent to inorganic sulfate and 55 per cent to cystine (cf. foot-note 2).

‡ Calculated according to Table IV (Periods A49 and A49a).

apparently derived from the catabolism of methionine (and pre-formed cysteine).

The results of the egg albumin experiment again indicate that one of the pathways of methionine catabolism is its conversion into cysteine, and that the cystine excreted in cystinuria is derived mainly from dietary methionine.

<sup>4</sup> Such calculations are significant only for protein superimposition experiments of short duration, rather than for cases in which a higher protein level is established over longer periods of time.

We are indebted to the patient, Mr. L. P., for his cooperation which made these studies possible.

#### SUMMARY

1. Superimposition experiments with methionine in a case of cystinuria showed, in agreement with Lewis *et al.*, that the excretion of extra cystine diminishes as the protein content of the diet is increased.

2. The distribution of the sulfur is reported for a preparation of denatured crystalline egg albumin.

3. Feeding experiments with egg albumin showed that cystine and methionine (and cysteine) were catabolized both qualitatively and quantitatively in the same way as when they were administered in the form of the free amino acids. This is in agreement with the results of the feeding experiments with casein and lactalbumin.

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## CYSTINURIA

### IX. THE METABOLISM OF LACTALBUMIN AND OF REDUCED LACTALBUMIN\*

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Experiments in cystinuria have shown that extra cystine is produced by administration of methionine, cysteine, and homocysteine, but not of cystine, homocystine, and glutathione (1-3). The results with the free amino acids were duplicated with respect to methionine and cystine by feeding experiments with three proteins: casein, egg albumin, and lactalbumin (4, 5), and it appeared that the cystine and methionine of the proteins were catabolized in the same way as the free amino acids. However, the behavior of cysteine as part of a protein molecule could not be readily investigated with food proteins, since they contain only small amounts of cysteine. It was therefore necessary to prepare a reduced protein.

Lactalbumin was chosen for this purpose, since it contains about equal amounts of cystine and methionine and only traces of pre-formed cysteine. The feeding experiments with lactalbumin and reduced lactalbumin are reported in this paper, and it will be seen that the excretion of extra cystine is much greater after the feeding of reduced lactalbumin. Cystine and cysteine as part of the same protein molecule behave as two different amino acids with separate catabolic pathways.

\* This report is from a dissertation submitted by Beatrice Kassel in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.



*Case History and Diet*—The experiments were carried out on cystinuric patient A (cf. (1, 5)); the dietary conditions were the same as those described in Paper VIII (5) of this series.

*Preparation and Analysis of Reduced Lactalbumin*—We are indebted to Dr. G. C. Supplee for the lactalbumin (Labco 7-HAAX) used in these experiments.

Reduction of lactalbumin in 500 mg. batches according to the technique of Mirsky and Anson (6) gave preparations which, on hydrolysis, were found to contain about 1 per cent cysteine (*i.e.* a reduction of about one-third of the cystine).

After considerable experimentation, the following technique was developed which yielded preparations in which about 80 per cent of the cystine was reduced. A 500 cc. brown bottle was fitted with a 3-hole rubber stopper carrying a separatory funnel, a gas inlet tube reaching to the bottom, and a gas outlet tube. Not more than 10 gm. of lactalbumin was ground with water and transferred to the bottle, a total of 100 cc. of water and a few drops of caprylic alcohol being used. A solution of 5 cc. of freshly distilled thioglycolic acid in 20 cc. of water was neutralized in ice under  $N_2$  to pH 7 with 2  $N$  KOH, diluted to 75 cc., and added through the funnel, with  $N_2$  passing through the bottle.<sup>1</sup> The stream of gas was continued while the mixture was adjusted to pH 7.2; bromthymol blue and phenol red were used as external indicators. The connections were closed and the bottle shaken vigorously in a mechanical shaker for 16 hours; the room temperature was about 30° when these experiments were carried out. A stream of  $N_2$  was again passed through the bottle and 18 cc. of a 1:1 solution of freshly distilled trichloroacetic acid added through the separatory funnel. The mixture was transferred to a 200 cc. bottle, centrifuged, and washed twelve times as follows: seven times by suspending the solid in 150 cc. of water and adding 15 cc. of 1:1 trichloroacetic acid, once with 150 cc. of acetone containing 0.15 per cent HCl and 1 per cent trichloroacetic acid, twice with the same amount of acetone containing only 0.15 per cent HCl, and finally twice with about 170 cc. of petroleum ether.<sup>2</sup> The protein was dried in the centrifuge bottle *in vacuo* ( $CaCl_2$ , NaOH, paraffin),

<sup>1</sup> On addition of the thioglycolic acid, the solution takes on a violet color, which gradually increases, but disappears on acidification with trichloroacetic acid.

<sup>2</sup> The washing with petroleum ether is necessary to remove acetone, which

and drying in a desiccator<sup>3</sup> continued after powdering until free from petroleum ether.

A considerable number of 10 gm. batches can be reduced at one time, and can be allowed to stand for a while in the closed bottles. We found it inadvisable, however, to carry out centrifuging and washing with more than two batches at a time, since speed is essential in obtaining a product with a high degree of reduction.

The preparations of reduced lactalbumin contained 4.8 to 5.2 per cent moisture, and traces of ash and HCl. Their content of total S, methionine, and cystine plus cysteine was practically the same as that of lactalbumin (*cf.* Table I). Reduced lactalbumin contained only traces<sup>4</sup> of thioglycolic or dithiodiglycolic acid; the specific color test (10) for these substances was always negative.

The yield of reduced lactalbumin was about 97 to 98 per cent, the greater part of the loss being mechanical. However, on combining the three acetone washings, a small amount of a snow-white, fluffy precipitate settled out. From 190 gm. of lactalbumin, a total of 3 gm. of this more soluble fraction was obtained. This fraction contained 1.71 per cent of total S, 3.1 per cent methionine, 2.9 per cent cysteine, 0.9 per cent cystine, and not more than a trace of thioglycolic acid. Methionine, cystine, and cysteine accounted for 98 per cent of the sulfur.

The total S in lactalbumin and in the various preparations of reduced lactalbumin was determined by the Pregl-Saschek method (11), methionine and cystine plus cysteine by the Baernstein method (8), and cystine and cysteine separately by the Folin photometric method (7). The Sullivan method was also used, but the results could not be interpreted quantitatively, since this method is not suitable for the analysis of mixtures of cystine and cysteine (*cf.* (8)). In some preparations of reduced lactalbumin cysteine was estimated by letting the protein react with dithiodiglycolic acid (at about pH 4) and determining photometrically (7, 12) the amount of thioglycolic acid formed.<sup>5</sup> The deter-

<sup>3</sup> If the reduced protein is dried at higher temperature, even *in vacuo* at 78°, some reoxidation of  $-SH$  takes place.

<sup>4</sup> Not more than 0.1 per cent of thioglycolic acid, calculated from the value of  $H_2S$  sulfur in the Baernstein determination (8). Dithiodiglycolic acid yields about 80 per cent of its S as  $H_2S$  during HI digestion (9).

<sup>5</sup> The method has not given satisfactory results with denatured crystalline egg albumin.

mination of —SH groups in the reduced proteins yielded slightly higher values for cysteine than were obtained after hydrolysis. This is not unexpected, since losses of cysteine are apt to occur during acid hydrolysis owing to humin formation (13). The hydrolysis of the proteins was carried out with 6 N HCl under N<sub>2</sub> for 6 to 8 hours in an oil bath at 130°, and the photometric deter-

TABLE I  
*Analysis of Lactalbumin (Labco 7-HAAX) and Reduced Lactalbumin (Batch 17)*

	Lactalbumin	Reduced lactalbumin*
	<i>per cent</i>	<i>per cent</i>
Nitrogen.....	14.6	14.0
Total S.....	1.41	1.43
Methionine S.....	0.60	0.58
Cystine S.....	0.83	0.14
Cysteine ".....	0	0.67
Methionine.....	2.8	2.7
Cystine.....	3.1	0.53
Cysteine.....	0	2.5
	<i>ratio</i>	<i>ratio</i>
N to S.....	10	10
" " methionine S.....	24	24
" " cystine S.....	18	100
" " cysteine ".....		21
Methionine S to cystine S.....	0.7	4
" " + cysteine S to cystine S.....		9
	<i>per cent of total S</i>	<i>per cent of total S</i>
Methionine S.....	43	41
Cystine S.....	59	10
Cysteine ".....		47

\* Cf. (7).

minations completed within 1 hour, because reoxidation of cysteine occurs if the hydrolysate is allowed to stand.

Batch 17 of reduced lactalbumin was fed to the patient; some details regarding its analysis have been presented previously (7) and the results are summarized in Table I.

*Metabolic Observations*—The procedure, methods, and basal

TABLE II  
*Metabolic Observations*

The values are given in gm.

Period No.	Date	Substance fed (50 gm. on date indicated)	Urine											
			Nitrogen		Creatinine		Sulfur					Cystine		
			Total	Urea	Pre- formed	Total	Total	Inor- ganic SO <sub>4</sub>	Ethe- real SO <sub>4</sub>	Neutral		Folin photo- metric method		
										Total	Cystine		Unde- ter- mined	
A54*	Aug., 1937 2-3		8.1	6.1	1.48	1.61	0.67	0.25	0.04	0.38	0.27	0.11	1.02	1.00
A55	4	Lactalbumin "	10.4	8.4	1.48	1.61	0.99	0.53	0.03	0.43	0.30	0.13	1.13	1.06
	11.8		9.3	1.58	1.66	1.01	0.54	0.03	0.44	0.33	0.11	1.22	1.13	
A55a	6		10.1	7.8	1.63	1.75	0.79	0.32	0.02	0.45	0.32	0.13	1.21	1.14
	7		8.4	6.4	1.58	1.64	0.64	0.26	0.03	0.35	0.27	0.08	1.00	1.00
A56*	8-10		8.1	6.0	1.54	1.64	0.68	0.28	0.02	0.38	0.28	0.10	1.03	1.02
A57	11	Reduced lactalbumin "	11.0	8.3	1.61	1.79	0.89	0.36	0.03	0.50	0.36	0.14	1.33	1.20
	12		11.5	8.8	1.52	1.76	1.03	0.46	0.04	0.53	0.40	0.13	1.47	1.34
A57a	13		9.5	7.3	1.58	1.72	0.79	0.34	0.03	0.42	0.32	0.10	1.18	1.14
	14		9.8	7.2	1.45	1.61	0.74	0.28	0.03	0.43	0.30	0.13	1.13	1.12
	15		8.4	6.3	1.58	1.70	0.72	0.29	0.03	0.40	0.29	0.11	1.08	1.09
A58	16		8.6	6.7	1.54	1.65	0.69	0.28	0.03	0.38	0.28	0.10	1.05	1.03

\* Average per day.

level of N and S intake were the same as described in Paper VIII (5). The metabolic data are presented in Table II. Throughout the experiments there was little change in the volume of the urine. There was a slight rise in the excretion of  $\text{NH}_3$  during the feeding of lactalbumin (Period A55); a rise from 0.3 to 0.6 gm. of  $\text{NH}_3\text{-N}$  per day was observed during the feeding of reduced lactalbumin (Period A57), probably due to the traces of HCl present in reduced lactalbumin. The values for volume,  $\text{NH}_3\text{-N}$ , undetermined N, and the figures for cystine obtained by the regular Sullivan method (*cf.* (5)) were omitted from Table II. Tests for sulphydryl compounds in the urine were negative throughout the experiments.

*Creatinine Excretion*—Both during control and experimental periods there were only minor variations in the excretion of pre-formed creatinine, indicating conditions satisfactory for metabolic experimentation. Ingestion of 100 gm. of lactalbumin or reduced lactalbumin did not result in a rise in the creatinine output.

*Lactalbumin (Periods A55 and A55a)*—As in the previous experiment ((5) Periods A26 and A26a), the ingestion of 100 gm. of lactalbumin resulted in a considerable increase in the excretion of inorganic sulfate and a small rise in that of cystine. In the present experiment, cystine returned to the basic level on the 2nd day of the adjustment period (No. A55a), while in the former experiment, on a lower basal N intake, the excretion of cystine continued at a slightly elevated level for several days ((5) Period A26a). This more rapid return to the control level is similar to the findings of Lewis, Brown, and White (3) on the difference in the behavior of the sulfur amino acids when superimposed on basal diets of varying N content. The excretion of total extra N and S (*cf.* Table III, calculated as described previously (4)) was greater in the present experiment (58 and 52 per cent respectively as compared to 35 and 48 per cent in the earlier experiment). Consequently, the N:S ratios of the lactalbumin fed, catabolized, and "stored" were 10, 12, and 9, while these ratios were 10, 8, and 13 in the former experiment (*cf.* (4) Table IV). The difference in the ratios is mainly due to greater excretion of extra N on the higher basal level of the present experiment, while the excretion of extra S was only moderately increased.

The partition of the extra S is reported in Table IV. Compared

to the former experiment on lactalbumin, inorganic sulfate accounts for a greater part of the extra S (81 instead of 67 per cent), while cystine S accounts for somewhat less (16 instead of 20 per cent). The results of the two experiments on lactalbumin therefore indicate that, at different levels of basal N intake, the behavior of cystine and methionine as components of lactalbumin is similar to that of the free amino acids (*cf.* (3, 4)). The S partition in the present experiment on lactalbumin (Periods A55 and A55a) was calculated on the basis of the results obtained with methionine at

TABLE III  
*Extra Nitrogen and Sulfur in Experimental Periods*

Period No.	Substance fed (100 gm.)	Amount of extra N			Amount of extra S		
		Fed		Excreted	Fed		Excreted
		gm.	gm.	per cent	gm.	gm.	per cent
A55, A55a	Lactalbumin	14.6	8.5	58	1.41	0.73	52
A57, A57a	Reduced lactalbumin	14.0	8.7	62	1.43	0.71	50

TABLE IV  
*Extra Cystine and Partition of Extra Sulfur in Experimental Periods*

Substance	Total S	Extra S excreted as						Extra cystine ex- creted
		Inorganic SO <sub>4</sub> -S		Undetermined neutral S		Cystine S		
		gm.	per cent	gm.	per cent	gm.	per cent	
Lactalbumin (A55, A55a).....	0.73	0.59	81	0.02	3	0.12	16	0.45
Reduced lactalbu- min (A57, A57a).	0.71	0.34	48	0.09	13	0.28	39	1.05

the higher basal N intake ((5) Table IV; the method of calculation was described previously). Table V shows that the partition of the extra S calculated is in agreement with that actually found. It appears, therefore, that during the metabolism of lactalbumin, no appreciable reduction of cystine to cysteine occurs, since in both experiments on lactalbumin all of the extra cystine excreted was apparently derived from the catabolism of methionine.

*Reduced Lactalbumin (Periods A57 and A57a)*—The ingestion of 100 gm. of reduced lactalbumin resulted in a considerable

increase in the excretion of cystine and a small rise in that of inorganic sulfate (*cf.* Table II). In contrast to the preceding experiment with lactalbumin, the excretion of extra cystine and of extra S continued for several days in the adjustment period (Table II, Period A57a). Since both proteins contained the same amount of total S and methionine S, and were superimposed upon the same basal diet, the different rate of excretion of extra S and extra cystine after the feeding of reduced lactalbumin and lactalbumin is apparently associated with their content of cysteine and

TABLE V

*Lactalbumin and Reduced Lactalbumin Partition of Extra Sulfur, Calculated and Found*

The values are given in gm.

Partition of extra S calculated from catabolism of	Inorganic SO <sub>2</sub> -S	Undetermined neutral S	Cystine S	Cystine
Lactalbumin (Periods A55 and A55a)				
0.43 gm. cystine S* +	0.40	0.03	0.00	0.00
0.31 " methionine S*	0.13	0.05	0.13	0.49
Calculated	0.53	0.08	0.13	0.49
Found ( <i>cf.</i> Table IV)	0.59	0.02	0.12	0.45
Reduced lactalbumin (Periods A57 and A57a)				
0.07 gm. cystine S* +	0.06	0.01	0.00	0.00
0.34 " cysteine S* +	0.15	0.00	0.19	0.70
0.29 " methionine S*	0.12	0.05	0.12	0.46
Calculated	0.33	0.06	0.31	1.16
Found ( <i>cf.</i> Table IV)	0.34	0.09	0.28	1.05

\* For calculations *cf.* (5) Table V.

cystine, respectively. The behavior of reduced lactalbumin and lactalbumin is similar to that of cysteine and cystine, the extra S derived from the catabolism of the latter being excreted more rapidly (1, 3).

The excretion of extra N and extra total S was almost identical after reduced lactalbumin and lactalbumin (*cf.* Table III). The N:S ratios of reduced lactalbumin fed, catabolized, and "stored" were 10, 12, and 7 (10, 12, and 9 with lactalbumin).

However, the partition of the extra S (Table IV) after the feeding of reduced lactalbumin was quite different from the partition after lactalbumin. The excretion of extra cystine after reduced lactalbumin was more than twice as much as after lactalbumin (1.05 gm. instead of 0.45 gm., corresponding to 39 and 16 per cent of the total S, respectively). Inorganic sulfate accounted for 48 per cent of the total S (81 per cent after lactalbumin).

These data make it evident that the excretion of extra cystine and the partition of the extra S are dependent on the distribution of the S between methionine, cysteine, and cystine in these two proteins. Since the total S and methionine contents of the two proteins are the same, the greater excretion of cystine after reduced lactalbumin is associated with its cysteine content. This is in line with the observations on the metabolic behavior of cystine and cysteine, which showed that only the latter yielded extra cystine (1, 3). The agreement between the partition of the extra S calculated (*cf.* (5) foot-note 3) and found (*cf.* Table V) furnishes additional evidence.

The experiments with casein (4), lactalbumin ((4), and Pe-riod A55), and crystalline egg albumin (5) have shown that cystine and methionine fed as constituents of these proteins are catabolized both qualitatively and quantitatively in the same way as the free amino acids, and in the same ratios in which they are present in the individual proteins. The experiment with reduced lactalbumin furnishes similar evidence with respect to cysteine, and also supports the previous conclusion that little if any reduction of cystine to cysteine occurred during the catabolism of these proteins.

The experiments with the free amino acids (1) indicated that cystine and cysteine should be considered as two individual and separate amino acids. The results with reduced lactalbumin show that cystine and cysteine, also as part of the same protein, may behave as two different amino acids with separate catabolic pathways.

Reduced glutathione fed to cystinuric patients is, unlike cysteine, almost completely oxidized to inorganic sulfate (1, 14). It was therefore concluded (1) that the metabolic behavior of an amino acid may vary markedly, depending upon whether it is catabolized as the free amino acid or in combined form as a pep-



tide. The present experiments would seem to confirm this view, if only free cysteine is absorbed after the ingestion of reduced lactalbumin. However, if cysteine is also absorbed in peptide form, it would appear that the metabolic behavior of cysteine peptides is the same as that of cysteine, glutathione being the exception.

The results of the experiments with reduced lactalbumin demonstrate that the cystine excreted in cystinuria is derived from methionine and preformed cysteine.

We are indebted to the patient, Mr. L. P., for his cooperation, which made these studies possible.

#### SUMMARY

1. The preparation and analysis of reduced lactalbumin are reported; about 80 per cent of the cystine of the original lactalbumin was reduced to cysteine.

2. The metabolism of lactalbumin and of reduced lactalbumin was investigated in a case of cystinuria. The patient was on a basal diet of somewhat greater N content than that used in a previous lactalbumin experiment.

3. The results of the two experiments with lactalbumin indicate that, at different levels of basal N intake, the metabolic behavior of methionine and cystine as components of a protein is similar to that of the free amino acid.

4. A larger amount of extra cystine was excreted after reduced lactalbumin than after lactalbumin.

5. The total S catabolized was accounted for by the catabolism of methionine, cysteine, and cystine. Under the conditions of the experiments, cysteine, as well as methionine and cystine, fed as a constituent of reduced lactalbumin and of lactalbumin, was catabolized qualitatively and quantitatively in the same way as the free amino acid.

6. The experiment confirms the previous conclusion that little if any cystine is reduced to cysteine during the enzymatic splitting and catabolism of the proteins investigated.

7. Cystine and cysteine, as part of the same protein, behave as two different amino acids with separate catabolic pathways. There

are probably separate mechanisms for the oxidation of S—S and —SH compounds.

8. The results indicate that the cystine excreted in cystinuria is derived from methionine and preformed cysteine.

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## THE DISTRIBUTION OF THE SULFUR IN CASEIN, LACTALBUMIN, EDESTIN, AND PAPAIN\*

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Methods for the determination of cystine, cysteine, methionine, and sulfate in proteins have been discussed in recent publications (1-3). A system of analysis has been developed in which each of these constituents is determined by at least two independent methods. In this paper, a comparison of the results obtained by the various methods and some experiments on the effect of the conditions of hydrolysis on the cystine determinations are presented. Hydrolysis with HCl gives satisfactory results, but for different proteins the optimum time of hydrolysis varies. Hydrolysis with  $\text{H}_2\text{SO}_4$  has not proved suitable for cystine determinations, particularly by the Sullivan method. The results with reduced lactalbumin make it obvious that the Sullivan method cannot be used for protein hydrolysates which contain a mixture of cystine and cysteine.

The sulfur distribution of a number of proteins is reported. With casein, lactalbumin, and reduced lactalbumin it is possible to account for all of the sulfur, while with edestin and a preparation of papain considerable amounts remain undetermined.

*Hydrolysis and Methods*—For the cystine determinations, the proteins were digested in an inert atmosphere ( $\text{CO}_2$  or  $\text{N}_2$ ) with 6 N HCl, 6 N  $\text{H}_2\text{SO}_4$ , or 42 per cent formic acid in 20 per cent HCl

\* This report is from a dissertation submitted by Beatrice Kassell in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

(4) at 130° for varying lengths of time.<sup>1</sup> HI digestion for the Baernstein determinations was carried out as described previously (3).

The HCl and H<sub>2</sub>SO<sub>4</sub> digests were filtered from the humin and analyzed as soon as the hydrolysis was completed, since it was noted that losses of cysteine may occur if the hydrolysate is allowed to stand.

The determinations by the photometric method (cystine and cysteine separately), by the Baernstein method (methionine as volatile iodide and as homocysteine, cystine plus cysteine, sulfate as H<sub>2</sub>S), and the gravimetric determination of sulfate were carried out as described previously (1, 3).

For the Sullivan method, the procedure described by Brand, Harris, and Biloon (5) was followed, but 10 seconds<sup>2</sup> were allowed between the addition of naphthoquinone sulfonate and sulfite. Suitable aliquots of the hydrolysates were neutralized to pH 7 shortly before the determination. The recent modification recommended by Sullivan and Hess (8) for the determination of cystine in urine was tried, but the results indicated that it is not advisable to use this modification with protein hydrolysates. In proteins containing cysteine, the "cystine" determination by the Sullivan method is too high, since cysteine develops about 1.5 times (9, 10) as much color as cystine (*cf.* "Reduced lactalbumin").

In a number of instances, HCl or H<sub>2</sub>SO<sub>4</sub> hydrolysates were treated with cuprous chloride (11). The results obtained both with the photometric and Sullivan methods on solutions of these precipitates after removal of copper (*cf.* (1)) were consistently low (70 to 80 per cent) and these values are not reported.

Total S was determined by the Pregl method as described by Saschek (12), except that the BaSO<sub>4</sub> was ignited. Up to 60 mg. of protein, distributed between two boats, can be burned in the Pregl apparatus without difficulty. About 1.5 cc. of Pregl's peroxide solution are used on the spiral and the combustion is allowed to proceed slowly for 1 to 2 hours.

<sup>1</sup> In some experiments, the CO<sub>2</sub> passed from the HCl hydrolysate into an absorber containing a 20 per cent solution of CdCl<sub>2</sub>, but there was never any evidence of H<sub>2</sub>S formation.

<sup>2</sup> The 10 second interval was originally recommended by Sullivan (6). Later Rossouw and Wilken-Jorden (7) studied the timing of the Sullivan reaction in detail.

A direct determination of —SH groups in the unhydrolyzed protein (*cf.* (13–15)) was carried out only with reduced lactalbumin. The protein was treated with a large excess of dithiodiglycolic acid at pH 4.0 and room temperature, being stirred with N<sub>2</sub> for 10 to 15 hours; the thioglycolic acid formed was determined photometrically (1, 2) after precipitation of the protein by trichloroacetic acid.

The results are reported in Tables I and II.

TABLE I  
*Effect of Hydrolysis on Cystine Determinations*

The values are the average of determinations on two to four hydrolysates.

Hydrolysis		Lactalbumin			Edestin; cystine	
Acid	Time	Cysteine	Cystine	Cystine, Sullivan method	Photo- metric method	Sullivan method
		Photometric method				
	hrs.	per cent	per cent	per cent	per cent	per cent
6 N HCl	4				1.27	0.85
6 " "	6	0	2.87	2.68	1.21	1.19
6 " "	6	0	3.06*			
6 " "	8	0.27	2.80	3.12	1.20	1.03
6 " "	8	0.02*	3.06*			
6 " "	15	0.23	2.46	2.28		
6 " "	48	0	2.87	(2.90)†		
6 " H <sub>2</sub> SO <sub>4</sub>	8	0	2.79	1.96	1.17	0.61
6 " "	15	0	2.76	1.95	1.19	0.77‡
20% HCl in 42% HCOOH	8	0	3.18	2.90		
	15	0	3.13	3.12		
	48	0	2.78	(3.52)†		

\* Micromodification.

† Hydrolysates strongly colored; values therefore too high.

‡ Approximately 1.0 per cent of cystine was found by the Sullivan method in cuprous chloride precipitates of the same hydrolysates.

*Humin Formation*—Humin formation during hydrolysis is frequently associated with losses of sulfur. For edestin, gliadin, and wool, Bailey (16) found that none of the methionine S is retained by the humin. On the other hand, Lugg (17) has shown that serious losses of cysteine occur owing to humin formation, while cystine is hardly affected. It therefore seems justified to

S retained by the humin. For this purpose, a sulfur determination according to Pregl's method is carried out on the thoroughly washed humin.

During HI hydrolysis there is no loss of cysteine due to humin

TABLE II

*Sulfur Amino Acids and Distribution of Sulfur in Various Proteins*

The values are given in per cent. Those given in parentheses are too high (see text).

Determina- tion No.	Substance and method of analysis	Casein (Labco)	Lactal- bumin (Labco)	Reduced lactal- bumin	Edestin	Papain
1	Cystine, photometric	0.34*	2.80*	0.53†	1.2†	3.2*
2	Cysteine, "	0.00*	0.27*	2.5†, ‡	0.00†	1.0*
3	Cystine, Sullivan		3.1*	(4.0)†	1.2†	
4	Cystine + cysteine, Baernstein	0.48	3.1	3.2	1.5	4.2
5	Methionine, volatile iodide	3.2	2.8	2.7	2.4	0.46
6	Methionine, homocysteine	3.0	2.8	2.3	2.3	0.41
7	Cystine S	0.09	0.75	0.14	0.39	0.85
8	Cysteine S	0.00	0.07	0.67	0.00	0.27
9	Methionine S	0.69	0.60	0.58	0.51	0.10
10	Sulfate S as H <sub>2</sub> S	0.01	0.01	(0.05)§	0.02	1.52
11	" " " BaSO <sub>4</sub>		0.00		0.01	1.54
12	Total S, Pregl	0.78	1.42	1.43	0.98	3.01
13	" " 7 + 8 + 9 + (10, 11)	0.79	1.43	1.44	0.91	2.76

\* 8 hour hydrolysis.

† 6 hour hydrolysis.

‡ Corrected (cf. (1)).

§ The H<sub>2</sub>S is due to traces of thioglycolic acid which is decomposed to about 80 per cent during HI digestion (Brand, E., and Kassell, B., unpublished experiments).

|| Total S was also determined with the Parr bomb.

formation. Therefore the values for cystine plus cysteine obtained by the Baernstein method are essentially correct (except for slight losses (1 to 2 per cent) due to decomposition<sup>3</sup> of cysteine to H<sub>2</sub>S).

<sup>3</sup> With the proteins so far investigated the decomposition does not exceed 2 per cent, except with crystalline insulin, in which about 7 per cent of the cystine S is decomposed (cf. (3, 18)).

A discrepancy between the photometric method and the Baernstein method is to be expected, if a protein on HCl hydrolysis yields considerable amounts of both cysteine and humin.

*Lactalbumin*—The lactalbumin (Labco 7-HAAX) was obtained through the courtesy of Dr. G. C. Supplee.

In the hydrolysis of lactalbumin only moderate amounts of humin are formed. The sulfur retained by the humin is negligible (the humin formed during the HCl hydrolysis (16 hours at 130°) of 3.2 gm. of lactalbumin contained only 0.004 per cent of S). Table I shows that this protein contained only very small amounts of cysteine.<sup>4</sup>

The optimum time for HCl hydrolysis is 8 hours, since the results by the photometric, Sullivan, and Baernstein methods check (Tables I and II). Digestion for less than 8 hours results in incomplete hydrolysis, as indicated by the low cystine values. Longer hydrolysis also gives low results, owing partly to cystine destruction (*cf.* photometric values) and partly to increased formation of substances which interfere with the Sullivan method.

Hydrolysis with H<sub>2</sub>SO<sub>4</sub> gives low results, particularly with the Sullivan method. This is apparently not due to cystine destruction, but to a tendency of the H<sub>2</sub>SO<sub>4</sub> to produce substances which interfere with this reaction (*cf.* "Edestin").

On hydrolysis with the HCl-formic acid mixture, cystine destruction seems to be somewhat decreased, in agreement with the findings of Miller and du Vigneaud (4), but the rate of hydrolysis is also decreased (*cf.* Table I).

All of the sulfur in this preparation of lactalbumin is accounted for as cystine, cysteine, and methionine (Tables II and III). A preparation of lactalbumin analyzed by Baernstein (19) apparently contained small amounts of inorganic sulfate, but he likewise accounted for all of the protein sulfur as cystine and methionine.<sup>5</sup>

*Reduced Lactalbumin*—Reduced lactalbumin was prepared by treatment of lactalbumin with thioglycolic acid as described previously (21). The analysis reported in Table II refers to the

<sup>4</sup> Another preparation of lactalbumin (used for feeding experiments in cystinuria) contained no cysteine.

<sup>5</sup> Baernstein's preparation (19) contained more cystine and less methionine than ours. Plimmer and Lowndes (20) analyzed a number of preparations of cow and of human lactalbumin and found variations in the total N, total S, methionine, and cystine. They did not account for the total S.



preparation of reduced lactalbumin used in feeding experiments in cystinuria (21).

With HCl the hydrolysis of the reduced protein seems to proceed somewhat faster than that of lactalbumin, since the same results were obtained after 6 and 8 hours (*cf.* (22)).

Hydrolysis with an HCl-formic acid mixture was not very satisfactory, because of the necessary evaporation of the formic acid.

Since reduced lactalbumin contained large amounts of cysteine, HCl hydrolysis resulted in some loss (about 10 per cent of the cysteine) due to humin formation. This loss, indicated by the discrepancy between the values obtained after HCl and HI hydrolysis (*cf.* "Lactalbumin"), could be confirmed by direct deter-

TABLE III  
*Percentage Distribution of Sulfur in Various Proteins*

Protein	Total protein S*	Protein S			
		Cystine	Cysteine	Methionine	Undetermined
Casein.....	0.78	12	0	88	0
Lactalbumin.....	1.42	53	5	42	0
Reduced lactalbumin....	1.38	10	49	42	0
Edestin.....	0.97	40	0	53	7
Papain.....	1.47†	58	18	7	17

\* Corrected for sulfate S.

† The possibility of the presence of organic sulfate S is not excluded.

mination of the —SH groups in the unhydrolyzed protein with dithiodiglycolic acid (*cf.* "Methods"). For instance, with another preparation of reduced lactalbumin, the photometric method indicated 1.28 per cent of cystine, and 1.53 per cent of cysteine, while the determination of —SH groups gave 1.68 per cent of cysteine, thus accounting for all of the cystine (3.1 per cent) of the original protein.

The values for "cystine" found by the Sullivan method were too high (reported in parentheses in Table II), varying from 4.0 to 4.3 per cent for preparations of reduced lactalbumin in which about 80 per cent of the original cystine (3.1 per cent) had been reduced.

Attempts to determine cystine plus cysteine as total cysteine

by the Sullivan method after reduction with zinc or titanous chloride (*cf.* (22)) were not successful, owing to difficulties in removing the reducing agent.

The total sulfur and  $\text{H}_2\text{S}$  sulfur (Table II) in reduced lactalbumin are slightly higher than in lactalbumin. This is apparently due to traces of thioglycolic acid, which is decomposed to  $\text{H}_2\text{S}$  during digestion with HI (to an extent of about 80 per cent<sup>6</sup>). As in lactalbumin, all of the protein S of reduced lactalbumin is accounted for as cystine, cysteine, and methionine (Tables II and III).

*Casein*—Casein (Labco) was obtained through the courtesy of Dr. G. C. Supplee. In view of its low cystine content, no detailed experiments on the effect of HCl hydrolysis were carried out. The casein contained no cysteine, and the results for cystine by the photometric and Baernstein methods are in reasonable agreement (Table II), although the value for cystine by the Baernstein method may be slightly high, owing to the possibility of titrating small amounts of homocysteine as cysteine (*cf.* (3)). All of the S of casein is accounted for as cystine and methionine (Tables II and III).

*Edestin (Hoffmann-La Roche)*—Table I shows that the optimum time for HCl hydrolysis is only 6 hours; hydrolysis for 8 hours resulted in lower values by the Sullivan method.

After  $\text{H}_2\text{SO}_4$  hydrolysis, the values by the Sullivan method were always considerably lower than by the photometric method. That this was due to the formation of interfering substances was clearly demonstrated by finding more cystine by the Sullivan method in the cuprous chloride precipitate (9) than in the original hydrolysate (*cf.* Table I, ‡ foot-note).

The values for cystine by the Baernstein method were almost 25 per cent higher than those by the photometric method.<sup>7</sup> This discrepancy<sup>8</sup> remains unexplained and subject to further investigation.

<sup>6</sup> Unpublished experiments with dithiodiglycolic acid.

<sup>7</sup> The value for cystine by the photometric method is in agreement with that found by Mirsky and Anson (14).

<sup>8</sup> Unpublished experiments point to cysteine sulfinic acid ( $\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{SO}_2\text{H}$ ) as a substance possibly responsible for the discrepancy, since we find that this compound is quantitatively reduced and determined as cysteine in HI digests. Its presence in HCl hydrolysates has no influence on the photometric cystine determination.

It can be seen from Tables II and III that the S in edestin is not accounted for by cystine, methionine, and sulfate, and that at least 7 per cent of the protein S remains undetermined (*cf.* (23)). Cystine destruction during HI digestion is excluded by the low value for  $H_2S$  sulfur, which is in agreement with the gravimetric sulfate determination.

*Papain*—Recent reports (24, 25) indicate that papain, as well as the related enzyme, ficin, is a protein. It was therefore thought desirable to determine the distribution of the S in papain, particularly since no such data are available in the literature. The enzyme was a purified, water-soluble preparation<sup>9</sup> of considerable activity, 1 mg. containing about 5 units (26).

The total S content was 3 per cent, of which about half was sulfate. The preparation contained about 3 per cent of cystine and 1 per cent of cysteine. (The values by the photometric and Baernstein methods checked.) There was a small amount of methionine present, which was determined both as volatile iodide and as homocysteine.

It can be seen from Tables II and III that the S in this preparation of papain is not accounted for by cystine, cysteine, methionine, and sulfate. About 17 per cent of the protein S (*cf.* Table III, † foot-note) remains undetermined.

#### SUMMARY

1. In determining the sulfur distribution of a protein, a number of methods are combined in the following system of analysis. Total sulfur is determined by the Pregl method or in a Parr bomb. Sulfate is determined gravimetrically after hydrolysis with HCl, and as  $H_2S$  after digestion of the protein with HI. Methionine is determined as volatile iodide and as homocysteine. The sum of cystine and cysteine is determined in an HI digest. Cystine and cysteine are determined separately in an HCl hydrolysate by the photometric method. The cysteine value is corrected for the humin sulfur, determined by the Pregl method. The more specific, but less precise, Sullivan method is used as a check on the cystine determination.

<sup>9</sup> Obtained through the courtesy of the Nippon Ferment Industrial Company.

2. Optimum conditions for HCl hydrolysis vary for different proteins.  $\text{H}_2\text{SO}_4$  hydrolysis is not recommended.

3. The regular Sullivan method can be used for cystine determinations in proteins, only if the absence of cysteine in the hydrolysate has been established.

4. All of the sulfur of casein, lactalbumin, and reduced lactalbumin can be accounted for as cystine, cysteine, and methionine, while in edestin and in a preparation of papain considerable amounts of the sulfur remain undetermined.

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# THE URICASE OF DOGS, INCLUDING THE DALMATIAN

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Interest in purine metabolism has taken on new impetus from the relation recently demonstrated between purines and certain oxidative enzymes (Keilin and Hartree (1)). In view of the uniquely high excretion of uric acid by Dalmatian dogs, discovered by Benedict (2), and the inheritability of this metabolic anomaly according to Mendelian laws (Trimble and Keeler (3)), it was thought worth while to study oxidation of uric acid by dog tissues, particularly liver, *in vitro*. Experiments were carried out on one pure blood Dalmatian, six Dalmatian crosses of known ancestry, and two dogs of mixed but unknown ancestry. All of the dogs, except one of those of unknown antecedents, were 6 months of age or under. The pure blood Dalmatian and one of the crosses excreted large amounts of uric acid—the others, small amounts.

In 1918, Wells (4) reported the presence of uricolytic activity in the liver of a single Dalmatian. He questioned whether or not a quantitative deficiency in uricase exists in the Dalmatian, but as far as we are aware, this question has not been answered. The present experiments were for the purpose of determining by quantitative observations whether a relation exists between the uricase content of the liver and the uric acid excretion by the animal.

## EXPERIMENTAL

The dogs were placed on a milk diet 2 days previous to the experiment. Immediately before the experiment, they were killed under chloroform anesthesia. (By comparing the uricase action of the liver of rats killed with and without the use of chloro-

form, it was found that the anesthetic was without effect on the uricase action.) The uricase action of the liver was determined in the following manner. The removed tissue was immediately ground in liquid air and suspended in Sørensen's borate buffer of pH 9.2, the optimum reaction for uricase action. 3 cc. of this tissue suspension, containing from 25 to 100 mg. of original tissue, were pipetted into Warburg vessels. 1 mg. of uric acid in the form of lithium urate was placed in the side arm of the vessel. All experiments were run in triplicate. After temperature equilibrium was established, the uric acid was mixed with the liver suspension. The  $\text{CO}_2$  produced was held as carbonate by the alkaline buffer and the consumption of oxygen was determined manometrically at  $38^\circ$ . The oxygen consumed by an equal amount of liver in the absence of uric acid was subtracted from the observed oxygen consumption. This provided data which permitted the estimation of the relative amount of uricase in the different samples of liver. Results have been expressed as the time required to half oxidize the uric acid present per 100 mg. of wet tissue. The relative uricolytic activities of the different livers were also expressed in terms of the reciprocal of the times required for half oxidation, the uricolytic activity of the pure blood Dalmatian being taken as unity. At the end of each experiment, uric acid determinations were carried out on tungstic acid filtrates of the liver suspension according to the method of Folin (5). With different preparations, the amount of oxygen used per mole of oxidized uric acid varied between 1 and 2 atoms. This fact, previously observed by Battelli and Stern (6), was explained by Keilin and Hartree (1) as oxidation of other substrates by hydrogen peroxide formed during the oxidation of uric acid. In each experiment, this was taken into account in the following manner in estimating the time required to half oxidize the uric acid. Half the total amount of oxygen taken up during the experiment divided by the fraction of uric acid destroyed during the same time was taken as the amount of oxygen corresponding to half oxidation of the uric acid. The time corresponding to this amount of uric acid oxidation is regarded as the half oxidation time.

Since preliminary experiments on rat liver showed that uricase activity depended largely on the size of the particles in suspension,

but that maximum activity could be consistently obtained by grinding frozen tissue in liquid air, the latter procedure was adopted. With the uniform suspensions so obtained, the rate

TABLE I

*Comparison of Rate of Uric Acid Oxidation with Varying Amounts of Liver*

Dog No.	Time of half oxidation of 1 mg. of uric acid	
	Liver, 50 mg.	Liver, 100 mg.
	min.	min.
F <sub>4</sub> *	46	23
F <sub>3</sub>	57	29
F <sub>2</sub>	44	21
F <sub>4</sub>	26	13

\* These numbers are identical with those used by Trimble and Keeler (3) in their study of the heredity of these animals.

TABLE II

*Comparison of Uric Acid Excretion of Dogs and Uricolytic Activity of Their Livers*

Dog No.	Breed	Age	Uric acid excretion	Time to half oxidation of uric acid per 100 mg. wet liver	Relative uricolytic activity
		mos.	mg. per kg. per day	min.	
P <sub>8</sub>	D	3.0	52	28	1.0
F <sub>4</sub>	DC	3.5	70	23	1.2
F <sub>3</sub>	"	5.3	10	29	0.9
F <sub>2</sub>	"	3.0	8	21	1.3
F <sub>4</sub>	"	6.2	7	13	2.2
F <sub>2</sub>	"	6.2	5	23	1.2
F <sub>1</sub>	"	2.8	4	21	1.3
X <sub>1</sub>	X	3-4		32	0.8
X <sub>2</sub>	"	Old		193	0.15

D designates pure blood Dalmatian; DC, Dalmatian-collie cross; X, unknown ancestry.

of uric acid oxidation was found to be proportional to the amount of tissue used. Data illustrating the proportionality of the rate of oxidation to the amount of tissue are given in Table I.



*Results and Comment*

In Table II the uricolytic activity of the liver of the dogs is compared with the uric acid excretion of the dogs on a purine-free diet. It will be seen that no quantitative relation exists between the uric acid excretion of the animals and the uricase content of their livers. The uricase content of the liver of the two dogs (Nos. P<sub>18</sub> and F<sub>4</sub>) which excreted large amounts of uric acid was of the same high magnitude as that of the dogs excreting little uric acid. The excessively high uricolytic activity of Dog F<sub>4</sub> in Table II is not understood. Other portions of the liver of the same dog studied in another experiment, Table III, showed an activity comparable to those of Dogs F<sub>3</sub> and F<sub>2</sub>. It is of interest that the old dog of unknown ancestry had the lowest uricolytic activity.

TABLE III

*Comparison of Uric Acid Oxidation by Ground Liver and Surviving Liver Slices Expressed As Mg. of Uric Acid per Mg. of Dry Tissue per Hour*

Dog No.	Ground liver	Sliced liver (average of 4 observations)
F <sub>3</sub>	0.057	0.027
F <sub>4</sub>	0.052	0.018
F <sub>2</sub>	0.049	0.025

The kidneys of three dogs and spleens of two dogs were examined but were found to contain no uricase. One of these, Dog P<sub>18</sub>, excreted large amounts of uric acid, the others, Dogs F<sub>1</sub> and F<sub>8</sub>, small amounts. The muscle of one dog, No. F<sub>8</sub>, with low uric acid excretion was also examined and found to be without uricolytic activity. In passing, it may be observed that, consistent with the findings of others, a similar experiment carried out on a specimen of fresh human liver revealed no uricolytic activity.

In view of the high uricase activity of the liver of all dogs studied, the question of why Dalmatian dogs excrete large amounts of uric acid cannot be attributed to the absence of this enzyme system. In an attempt, therefore, to obtain information on the cause of the apparent inactivity of uricase *in vivo*, the uricolytic action of surviving liver slices was compared with the oxidation

of uric acid by ground liver. The oxygen consumption of both the ground and sliced liver was determined in the presence and absence of glucose. The surviving liver slices gave normal values of  $Q_{O_2}$  for dog liver, indicating that the liver slices were respiring normally. Upon the addition of uric acid, the oxygen consumption of the ground liver increased greatly, while that of the liver slices did not increase significantly. These experiments were carried out on the livers of five dogs, one of which (No. P<sub>18</sub>) excreted a large amount of uric acid. Colorimetric determinations of the uric acid oxidized by the liver under the above conditions were made in the case of three of the dogs which excreted small amounts of uric acid. These results are shown in Table III, which shows that tissue slices were much less active in their oxidation of uric acid than ground tissue. This fact, as well as the observation that maximum uricase activity can be obtained only in extremely fine tissue suspensions, indicates that the enzyme is set free in active form by destroying the structure of the liver cells. This suggests that the inactivity of uricase *in vivo* may be due to the inaccessibility of the site of the uricase in the cell to the urate ions, although chemical inactivation of the enzyme in the intact cell is not excluded. However, our experiments offer no adequate explanation for the difference between the purine metabolism of the Dalmatian and other dogs.

#### SUMMARY

Livers of Dalmatian dogs excreting large amounts of uric acid are rich in uricase. No correlation was found between the amount of uricase present in the liver and the destruction of uric acid *in vivo*. Actively respiring liver slices were observed to have less uricolytic activity than ground liver suspensions.

No uricase was found in kidney, muscle, or spleen of the dogs examined.

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## THE SYNTHESIS OF ESTERS OF URSOLIC ACID

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Ursolic acid is a monohydroxytriterpene acid with the empirical formula  $C_{29}H_{48}(OH)COOH$  which occurs in the wax-like coating of the skin or cuticle of fruits and in the leaves of certain plants (1-4).

Various derivatives of ursolic acid have been synthesized (5-11). This communication deals with the preparation of monoacetylursolyl chloride and the corresponding homologous series of *n*-alkyl esters.

Monoacetylursolyl chloride was utilized by Sando (5) in the preparation of monoacetylmethylursolate but he did not isolate the acid chloride in a crystalline condition. By careful manipulation we have been able to obtain a crystalline product.

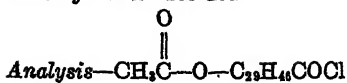
The acid chloride has been used as the starting product for the preparation of seven new *n*-alkyl esters. Together with the previously known methyl ester, they constitute the homologous series of *n*-alkyl esters through the *n*-octyl ester of monoacetylursolic acid.

### EXPERIMENTAL

*Preparation of Monoacetylursolyl Chloride*—To 22 gm. of monoacetylursolic acid were added slowly 44 cc. of cold thionyl chloride. After 12 hours (at room temperature) the excess of thionyl chloride was removed by distillation under reduced pressure. The residue was crystallized from hot hexane until a constant melting point was obtained. It was dried in an Abderhalden drier for 2 hours at  $111^{\circ}$  and 5 mm. over phosphorus pentoxide. The following constants were observed.

## 452      Synthesis of Esters of Ursolic Acid

Melting Point—200–201°



Calculated. Cl 6.90, saponification No. 325

Found.     " 7.02,                     "                     " 322

The identity of the monoacetylursolyl chloride was proved by its reaction with methanol to yield the known monoacetylmethylursolate of melting point 246°.

TABLE I  
*Esters of Monoacetylursolic Acid*

Ester	Yield	M.p.	[α] <sub>D</sub> <sup>25</sup>	Carbon		Hydrogen	
				Calculated	Found	Calculated	Found
	per cent	°C.	degrees	per cent	per cent	per cent	per cent
$\text{CH}_3\overset{\text{O}}{\underset{\parallel}{\text{C}}}\text{—O—C}_{29}\text{H}_{48}\text{COOC}_2\text{H}_5$	56	194	+60.8	77.5	76.5	10.3	10.8
$\text{CH}_3\overset{\text{O}}{\underset{\parallel}{\text{C}}}\text{—O—C}_{29}\text{H}_{48}\text{COOC}_3\text{H}_7$	77	173	+58.5	77.7	77.3	10.4	10.9
$\text{CH}_3\overset{\text{O}}{\underset{\parallel}{\text{C}}}\text{—O—C}_{29}\text{H}_{48}\text{COOC}_4\text{H}_9$	81	125–126	+54.5	77.9	77.8	10.5	10.9
$\text{CH}_3\overset{\text{O}}{\underset{\parallel}{\text{C}}}\text{—O—C}_{29}\text{H}_{48}\text{COOC}_5\text{H}_{11}$	42	110–111	+54.3	78.1	78.4	10.6	10.9
$\text{CH}_3\overset{\text{O}}{\underset{\parallel}{\text{C}}}\text{—O—C}_{29}\text{H}_{48}\text{COOC}_6\text{H}_{13}$	51	123–124	+54.8	78.3	78.5	10.7	10.5
$\text{CH}_3\overset{\text{O}}{\underset{\parallel}{\text{C}}}\text{—O—C}_{29}\text{H}_{48}\text{COOC}_7\text{H}_{15}$	47	93	+52.8	78.5	79.8	10.8	10.1
$\text{CH}_3\overset{\text{O}}{\underset{\parallel}{\text{C}}}\text{—O—C}_{29}\text{H}_{48}\text{COOC}_8\text{H}_{17}$	14	67	+51.5	78.7	78.5	10.9	10.6

The combustions were made for us by analysts in the laboratories for Organic Chemistry at Michigan State College and for Biochemistry at the University of Wisconsin.

*Preparation of Homologous Series of n-Alkyl Esters of Monoacetylursolic Acid*—The esters were prepared by treating monoacetylursolyl chloride (5) with the corresponding alcohols. In each case the alcohol was removed by distillation *in vacuo*. The residue was reacylated and recrystallized several times from ethanol until a constant melting point was obtained. The ethyl and hexyl esters were recrystallized from 95 per cent ethanol and the other esters from dilute ethanol. All of the compounds were obtained in a crystalline condition. The esters were dried in an Abderhalden drier for 2 hours at 111° and 5 mm. over phosphorus pentoxide, with the exception of the amyl and heptyl esters which were dried at 78° and 66° respectively. The octyl ester was dried at room temperature for 1 week at 5 mm. over phosphorus pentoxide. Crystallization became more difficult with the higher series owing to the increase of solubility of the ester in the solvent. The esters are soluble in xylene, benzene, hexane, chloroform, ether, ethylene dichloride, acetone, and toluene and insoluble in water. The physical constants and analyses of the esters are given in Table I.

#### SUMMARY

1. The preparation of monoacetylursolyl chloride is described.
2. The homologous series of *n*-alkyl esters through the octyl ester of monoacetylursolic acid has been synthesized.
3. The compounds were all obtained in crystalline condition and the physical constants and analyses are reported.

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# INFLUENCE OF YEAST-CONTAINING DIETS ON THE TOTAL FATTY ACIDS AND CHOLESTEROL CONTENT OF THE LIVERS OF INTACT AND PARTIALLY NEPHRECTOMIZED RATS\*

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During a study to determine the effect of yeast-containing diets on kidney hypertrophy in intact and partially nephrectomized rats, it was noted that the livers of some of the intact animals showed gross fatty changes on inspection, while the livers of partially nephrectomized rats appeared normal. It was felt that this observation warranted a study of the lipid content of the livers of intact and partially nephrectomized rats fed diets containing varying concentrations of dried yeast.

## Methods

Inbred rats of Wistar strain were maintained on a stock diet (Bal Ra<sup>1</sup>) until they were 60 days of age. At this time one group of animals was subjected to partial nephrectomy (1) which involved the removal of about 85 per cent of kidney tissue; another group of intact rats of the same age served as controls. These animals were placed on diets containing dried yeast<sup>2</sup> and designated as Diets Y20, Y30, Y40, Y60, and Y80 according to the percental concentration. A small group of intact rats was fed a control fat diet containing extracted meat in order to approximate the fat content of the yeast diets (Table I). All animals were sacrificed after being on the respective diets for 100 days.

\* This work was aided by a grant from the Penrose Fund of the American Philosophical Society.

<sup>1</sup> Purchased from Valentine's Meat Juice Company, Richmond.

<sup>2</sup> Kindly furnished by Standard Brands, Inc.



Analysis for total fatty acids and free and esterified cholesterol was begun immediately after removal of the liver under ether anesthesia. Wedges were removed from the different lobes and combined for analysis. For the determination of fatty acids, 4 to 6 gm. of liver were dried at 105° for 48 hours and then heated on a steam bath with 10 per cent alcoholic KOH under a reflux condenser for 4 hours. This solution was acidified with concentrated HCl and the fatty acids were extracted with petroleum ether. The fatty acids were weighed after evaporation of the solvent. For the determination of free and total cholesterol, about 0.4 gm. of liver was thoroughly ground in a mortar and transferred to a small piece of glass tubing; this was ejected into

TABLE I  
*Experimental Diets*

Diet.....	Y20	Y30	Y40	Y60	Y80	Control fat
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Yeast.....	20	30	40	60	80	5
Starch.....	54	44	34	14	5	52
Lard.....	17	17	17	17	6	14
Cod liver oil.....	5	5	5	5	5	5
Salt (Osborne and Mendel (2))...	4	4	4	4	4	4
Extracted beef.....						20
Nitrogen content.....	1.9	2.5	3.3	4.7	6.6	3.0
Fatty acid content.....	22.8	22.4	23.4	25.1	15.4	17.3

a 10 cc. volumetric flask containing an absolute alcohol and acetone mixture (1:1).<sup>3</sup> The extract was analyzed according to the procedure of Schoenheimer and Sperry (3, 4).

### *Results*

The concentration of the total fatty acids in the livers of individual intact and partially nephrectomized rats fed the yeast diets is shown in Fig. 1. In the intact animals it is seen that the livers of the group fed Diet Y20 have the greatest concentration of fatty acids and that there is a striking decrease in the fatty acid concentration as the yeast content of the diet is

<sup>3</sup> We are indebted to Dr. W. M. Sperry for this procedure.

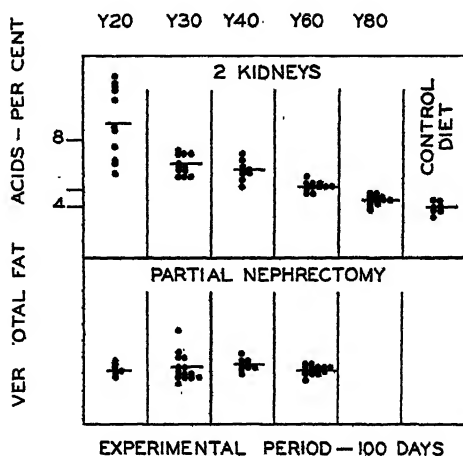


FIG. 1. Total fatty acids of wet livers of intact and partially nephrectomized rats fed diets containing various concentrations of yeast.

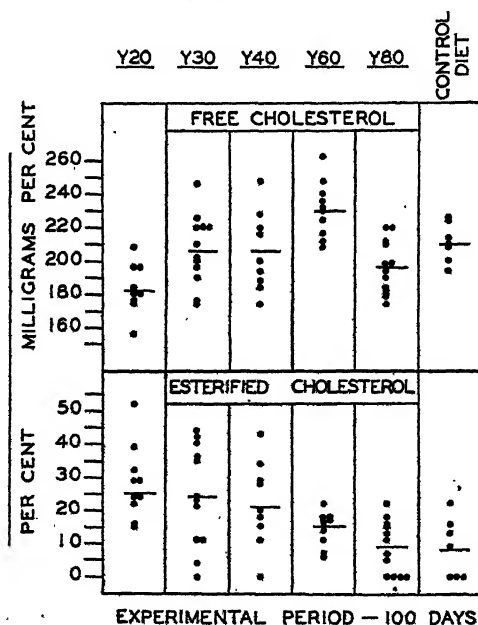


FIG. 2. Free and esterified cholesterol content of wet livers in intact rats fed diets containing various concentrations of yeast.

increased. The average values of fatty acids for the livers of the intact animals expressed in percentage of wet and dry weight, respectively, for the different diets are as follows: Diet Y20 9.0 and 27.2, Diet Y30 6.5 and 20.4, Diet Y40 6.1 and 19.2, Diet Y60 5.2 and 16.4, and Diet Y80 4.3 and 14.4; whereas in the partially nephrectomized rats the average percentage values are: Diet Y20 4.2 and 14.1, Diet Y30 4.3 and 14.7, Diet Y40 4.5 and 15.0, and Diet Y60 4.2 and 13.9. The constancy of the fatty acid values in the nephrectomized group is in striking contrast to those of the intact animals. Partial nephrectomy with its resulting renal insufficiency unquestionably influences lipid metabolism under the present experimental conditions. The average fatty acid concentration in the wet and dry liver of the control fat diet group is 3.9 and 14.4.

The individual values for the free and esterified cholesterol of the livers of intact rats are presented in Fig. 2. There is a marked variation in the individual values for the concentration of free and esterified cholesterol in the livers of the rats on the respective diets. The fatty acid concentration could not be correlated with the cholesterol fractions.

#### DISCUSSION

In this study, the intact rat had the highest concentration of fatty acids in the liver when on Diet Y20 and the percentage of fat decreased as the percentage of yeast in the diet increased. On the other hand the fatty acid content of the livers of partially nephrectomized rats was not affected by the yeast diets. These observations are difficult to explain. Yeast is rich in plant sterols, thiamine, and choline. Schoenheimer (5) and Breusch (6) have shown that plant sterols are not absorbed from the intestinal tract. McHenry (7) has shown that oral administration of thiamine to rats maintained on choline-free diets causes an increase in liver fat and Best and Huntsman (8) have demonstrated that fatty livers could be prevented by lipotropic substances such as choline. McHenry makes the assumption that thiamine and choline have antagonistic effects. If such an assumption is justifiable, it would seem plausible in the present investigation to postulate that in Diet Y20 the effect of the thiamine predominated over the choline effect and exerted its maximal effect, but

that as the concentration of yeast increased, the reverse effect occurred. If such a hypothesis is correct, the lack of fat accumulation in the livers of partially nephrectomized rats could be explained on the basis that the renal insufficiency produced by operation in some way interfered with the action of thiamine.

Reports from this laboratory (9, 10) have shown that the cholesterol and phospholipid concentrations of the blood plasma are affected as a result of renal insufficiency produced by partial nephrectomy in the rat. In these experiments further evidence that lipid disturbance in renal insufficiency is associated with primary kidney damage is presented.

#### SUMMARY

The total fatty acids and free and esterified cholesterol concentrations have been determined in the livers of intact and partially nephrectomized rats fed diets containing varying percentages of dried yeast (20, 30, 40, 60, and 80 per cent).

Intact rats develop fatty livers on these diets. The fatty acid concentration decreases progressively as the yeast content increases.

The livers of partially nephrectomized rats show no fatty changes on the yeast diets. These experiments show that renal insufficiency as produced by partial nephrectomy affects the lipid metabolism.

No consistent changes were noted for the free and esterified cholesterol of the livers of intact rats on the various yeast diets.

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# DETERMINATION OF SACCHAROIDS AND THE RELATIONSHIP OF THE SACCHAROID CONTENT OF THE BLOOD TO DIET AND DRUGS\*

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It is now well established that blood contains a small non-sugar reducing fraction. While several investigators have indicated the relative constancy of these saccharoid substances in most diseases (1, 2), still the literature contains no data concerning their status under various conditions existing in normal animals. This paper presents a study of the saccharoids during fasting and different dietary regimens and after the administration of certain drugs. Since the saccharoids have not as yet been fully defined chemically, their quantitative expression remains, for the time being, a function of the method used for their determination. In this study the Somogyi modification of the Shaffer-Hartmann cuprous titration reagent<sup>1</sup> for blood sugar (3, 4) was employed and a preliminary investigation was made of certain factors affecting the results of saccharoid determination with this reagent.

With the Shaffer-Hartmann cuprous titration technique, saccharoids may be estimated either (a) directly through analysis of saccharoid-containing filtrates prepared after yeast fermentation of the blood sample, as outlined by Somogyi (5) and Benedict

\* A preliminary report upon this work was presented before the Philadelphia Physiological Society and an abstract of this report has appeared (*Am. J. Med. Sc.*, **184**, 889 (1932)).

<sup>1</sup> During the course of the investigation it has been found, by periodic titration against standard dichromate solutions, that even 0.005 N  $\text{Na}_2\text{S}_2\text{O}_4$  may be preserved over a period of approximately 3 years when prepared in freshly boiled water and kept in dark bottles under toluene.

(6), or (b) indirectly through the difference obtained in the analysis of two types of filtrates, only one of which is saccharoid-containing.

In the direct determination of saccharoids we employed the Folin-Wu protein precipitation (7) of the fermented blood sample. It is of great importance to prepare accurately the prescribed 0.66 N  $\text{H}_2\text{SO}_4$ , since slight excesses in acidity were found to cause errors as high as 40 mg. per cent. The error probably arises in the liberation of saccharoids from the yeast cells. Blank analyses were performed upon the yeast suspension to which excesses of 0.66 N  $\text{H}_2\text{SO}_4$  ranging from 0.5 to 5.0 cc. were successively added to the routine Folin-Wu reagents. We obtained reducing values for the blank progressing from 14 to 40 mg. per cent in terms of glucose. Filtrates made upon yeast-fermented blood contain reducing substances equivalent to 20 to 40 mg. per cent, a range at which the Shaffer-Hartmann method is recognized to be relatively inaccurate. We, therefore, routinely concentrated our filtrates over a microburner to one-half the original volume. When employed with reagents, carefully calibrated against standard glucose solutions, and concentrated filtrates, the direct determination of saccharoids is extremely reliable. In experiments upon dogs a number of such analyses were performed upon a single animal during the course of 3 or 4 hours and the successive results were in close agreement.

For the indirect determination of saccharoids several procedures are now available for the preparation of filtrates designed to contain or to be relatively free from saccharoids.

All non-protein constituents in blood are ordinarily considered present in the Folin-Wu filtrates but Benedict (8) believed these give low values for thioneine. The latter introduced tungstomolybdic acid as a protein precipitant to obviate this error. Since the thioneine content of the blood is small, comparative blood sugar analyses upon the two filtrates should correspond approximately. We have found that in thirty-six such determinations, twenty-four checked within  $\pm 6$  mg. per cent, while in the remaining twelve the discrepancies varied from 7 to 45 mg. per cent. In our hands the results did not show any preponderance of higher values for total reducing substances upon the Benedict filtrate in comparison with the Folin-Wu filtrate. This finding is not necessarily opposed to Benedict's conclusion, since we have not used Benedict's blood sugar reagent (9).

Of the techniques utilizing zinc or copper protein precipitants to obtain saccharoid-free filtrates, we have followed that of Somogyi (10). Since the saccharoids are predominantly intracellular, Folin's "non-hemolyzed" filtrates (11) should yield closely approximate values. It was observed that sixteen of twenty-seven comparative analyses agreed within  $\pm 5$  mg. per cent and the remaining eleven showed discrepancies ranging from 8 to 26 mg. per cent. In general, values were somewhat higher upon the Folin "non-hemolyzed" filtrates. On the basis of the above comparative findings, we felt justified in preparing Folin-Wu filtrates for total reducing substances and Somogyi filtrates for glucose alone in the performance of indirect saccharoid analyses.

From theoretical considerations, one would expect that the determination of saccharoids upon a single blood specimen by both the direct and indirect techniques would give results in close agreement, but actually we have found no such correspondence, and we noted wide discrepancies, particularly with elevated blood sugars. In a study of blood drawn from fifteen cats (Table I) the direct method yielded values of 32 or 33 mg. per cent, while the indirect procedure gave results ranging from 26 to -90 mg. per cent. Blood from three dogs analyzed by the direct determination had a saccharoid content of 27 to 30 mg. per cent and by the indirect, 15 to -5 mg. per cent. We have designated as a "negative" saccharoid value a result in which the analysis of a Folin-Wu filtrate gave a lower figure than the analysis of a corresponding Somogyi filtrate. While the strikingly discrepant results were obtained upon samples with total blood sugar contents between 274 and 355 mg. per cent, still it is clear from the results upon the blood specimens of four cats and two dogs that even at moderately elevated blood sugars (105 to 155 mg. per cent) the indirect determinations were definitely low.

In order to rule out any physiological explanation for the low or even "negative" saccharoid values yielded by the indirect method, we performed determinations upon blood samples before and after the addition of glucose *in vitro*. The samples were obtained from normal unanesthetized dogs and were divided into two fractions, to one of which sufficient glucose was added to increase the total reducing values by 100 to 170 mg. per cent. In each of twelve such experiments (Table II) our results indicated a reduction of from 5 to 37 mg. per cent in the saccharoid content



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of that fraction to which glucose was added as compared with the determination upon the original blood.

The low saccharoid values by the indirect method, obtained at elevated blood sugar levels, may be due to excessive reduction figures upon the Somogyi filtrates, to deficient reduction figures

TABLE I

*Saccharoid Content of Cat and Dog Blood As Determined by Indirect and Direct Methods of Analysis*

The results are expressed in mg. per 100 cc.

		"Blood sugar"		Saccharoids		Remarks
		Folin-Wu filtrate	Somogyi filtrate	Indirect method*	Direct method, yeast	
Cat	1	174	162	12	32	
"	2	171	159	12	32	
"	3	153	148	5	33	Ether anesthesia
"	4	186	160	26	32	
"	5	258	348	-90		Ether anesthesia
"	6	254	261	-7		
"	7	105	103	2		
"	8	204	189	15		
"	9	174	154	20		
"	10	137	125	12		Amytal anesthesia
"	11	141	121	20		" "
"	12	168	161	7		Ether "
"	13	342	355	-13		" "
"	14	274	288	-14		" "
"	15	328	317	11		" "
Dog	1	155	160	-5	30	0.2 gm. glucose per kilo intracardially
"	2	125	110	15	29	1 gm. glucose per kilo intraperitoneally
"	3	201	198	3	27	2.5 gm. glucose per kilo intraperitoneally

\* Determination on Folin-Wu filtrate minus determination on Somogyi filtrate.

upon the Folin-Wu filtrates, or to both these factors. Upon repetition of these experiments with addition of known amounts of the glucose (Table III) it was found that in four of five instances the added glucose was recovered satisfactorily from the Somogyi filtrates, but recovery was deficient by from 8 to 31 mg. per cent

TABLE II

*Reduction in Saccharoid Values by Indirect Method upon Addition of Glucose to Blood Sample*

The results are expressed in mg. per 100 cc.

Dog No.	"Blood sugar"		Saccha- roids, in- direct method*	"Blood sugar," glucose added		Saccharoids	
	Folin-Wu filtrate	Somogyi filtrate		Folin-Wu filtrate	Somogyi filtrate	Indirect method*	Direct method, yeast
1	114	85	29	283	291	-8	
2	98	78	20	214	230	-16	
3	110	82	28	229	210	19	
4	114	87	27	255	245	10	
5	99	78	21	237	228	9	
6	107	79	28	247	224	23	
7	98	74	24	234	228	6	
8	86	59	27	239	239	0	
9	102	72	30	243	232	11	32
10	97	64	33	243	225	18	29
11	88	65	23	205	191	14	
12	87	65	22	193	181	12	25

\* Determination on Folin-Wu filtrate minus determination on Somogyi filtrate.

TABLE III

*Recovery of Glucose Added to Folin-Wu and to Somogyi Filtrates*

The results are expressed in mg. per 100 cc.

Dog No.	"Blood sugar"		Saccha-roids*	Glucose added	"Blood sugar," glucose added				Saccharoids	
	Folin-Wu fil-trate	So-mogyi filtrate			Folin-Wu filtrate		Somogyi filtrate		In-direct*	Yeast
					Calcu-lated	Found	Calcu-lated	Found		
1	98	72	26	140	238	219	212	215	4	30
2	79	61	18	140	219	202	201	193	9	26
3	98	78	20	147	245	214	225	230	-16	
4	98	74	24	151	249	234	225	228	6	
5	88	65	23	125	213	205	190	191	14	27

\* Determination on Folin-Wu filtrate minus determination on Somogyi filtrate.

upon the Folin-Wu filtrates. It is thus clear that the low and even "negative" saccharoid values obtained by the indirect method of determination must be attributed to deficient reduction figures

upon Folin-Wu filtrates, since there is a failure to recover quantitatively added glucose in analyses of such filtrates. This error is most manifest at elevated blood sugars but consultation of Tables I, II, and III indicates that even with blood samples of normal sugar content there is only approximate agreement between saccharoid determinations carried out by both the direct and indirect procedures.

As Somogyi (4) has reported that excess of salts, such as sodium sulfate and mercuric nitrate, influences the reduction by glucose of the Shaffer-Hartmann reagent, we investigated the action of tungstic acid in this respect in a preliminary search for the cause of the low values obtained on the Folin-Wu filtrates. We noted an average deficiency of 43 mg. per cent in reduction values upon glucose solutions ranging from 100 to 300 mg. per cent when these solutions were treated as blood and subjected to the Folin-Wu protein precipitation procedure before analysis. A corresponding treatment of glucose solutions with Somogyi reagents resulted in no significant change in reduction figures. This finding suggests that depressed reduction of the Shaffer-Hartmann reagent in the presence of tungstic acid or its salts is the explanation for the deficient reduction values we obtained on analysis of Folin-Wu filtrates. Efforts to carry out the experiments outlined above on glucose solutions made up in 10 and 20 per cent egg albumin, so as to simulate blood more closely, gave inconclusive results. It is important to point out that the depressant action of tungstate apparently is limited to the Shaffer-Hartmann blood sugar reagent, for when we substituted the Folin-Wu blood sugar reagent (12), we obtained quantitative recovery of glucose added to blood samples from which Folin-Wu filtrates were prepared and analyzed. Somogyi filtrates, on the other hand, proved unsatisfactory for analysis with the Folin-Wu blood sugar reagent, since a stable blue color failed to develop after the addition of the phosphomolybdic acid.

It is evident that in the determination of saccharoids, the indirect procedure, or the method of differential analysis of filtrates, cannot be employed indiscriminately. Results from its use will depend not only upon the saccharoid content, but also upon the presence of constituents in the filtrate which may affect the

particular blood sugar reagent employed. While the errors of the indirect procedure are chiefly manifested at high blood sugar levels, still we feel that in any critical study of the saccharoids the direct yeast procedure is the method of choice.

In the course of our experimental work saccharoid determinations by the direct procedure were performed upon dogs, cats, pigeons, and rats. In fourteen dogs saccharoids averaged 26.5 mg. per cent in terms of glucose, with extreme ranges of 22 to 30 mg. per cent. An average saccharoid content of 32 mg. per cent was found in four cats. A few analyses upon rats and pigeons showed a saccharoid content of about 30 mg. per cent in the former and 70 to 81 mg. per cent<sup>2</sup> in the latter. These values reveal an approximately similar quantity of saccharoids in the blood of dogs, cats, rats, and man, for Somogyi (5) has reported that human blood contains an average of 22.3 mg. per cent of reducing non-sugar substances, as determined by a yeast technique identical with that we have utilized.

In studying the effect of diet upon the blood saccharoid content, analyses were performed upon two dogs over periods of 4 and 7 months. The following diets were fed: (1) meat for 30 days; (2) high protein synthetic diet (13)<sup>3</sup> for 48 and 56 days; (3) low protein, high sugar synthetic diet (13) for 26 days; (4) high protein synthetic diet containing 25 per cent by weight of dried yeast for 13 days. The yeast regimen was employed because of its high glutathione content in an effort to increase this constituent of the saccharoids.

Our observations during the metabolism periods are presented in Figs. 1 and 2. It will be noted that the saccharoids in both

<sup>2</sup> Pigeon blood, in comparison with dog blood, is relatively concentrated. On the pigeon blood yielding a saccharoid value of 81 mg. per cent, the hematocrit reading was 66 per cent, in comparison with an average of approximately 45 per cent on our dogs. 81 mg. per cent is reduced to 55 mg. per cent when corrected on the basis of the above hematocrits; hence blood concentration does not account for the unusually high saccharoid content of pigeon blood.

<sup>3</sup> On the high protein diet 25 per cent of the calories was furnished by commercial casein, 30 per cent by sucrose, and 45 per cent by lard. In the low protein, high sugar diet approximately 5 per cent of the calories was supplied by casein, 50 per cent by sucrose, and 45 per cent by lard.

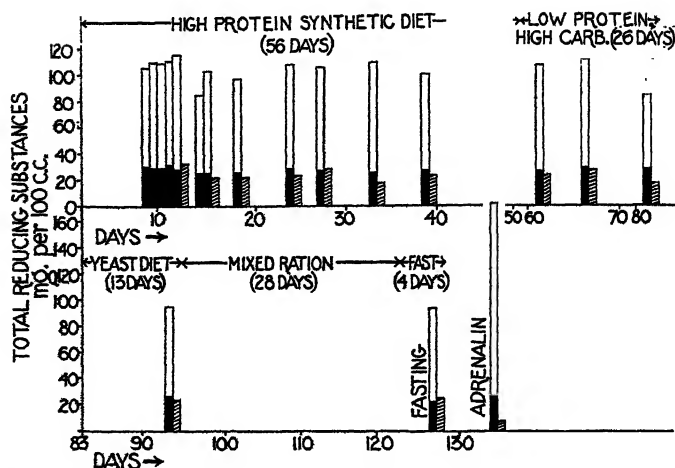


FIG. 1. Saccharoid analyses under various dietary regimens, fasting, and adrenalin administration. The total height of the columns represents magnitude of total reducing substances in the blood. The height of the solid columns represents the value for saccharoids obtained by the yeast fermentation procedure (the direct method). The height of the cross-hatched columns represents saccharoid values obtained by the difference between analyses upon Folin-Wu and Somogyi filtrates (the indirect method). The height of the clear columns represents the value for true sugar (the difference between total reducing substances and saccharoids by the direct method).

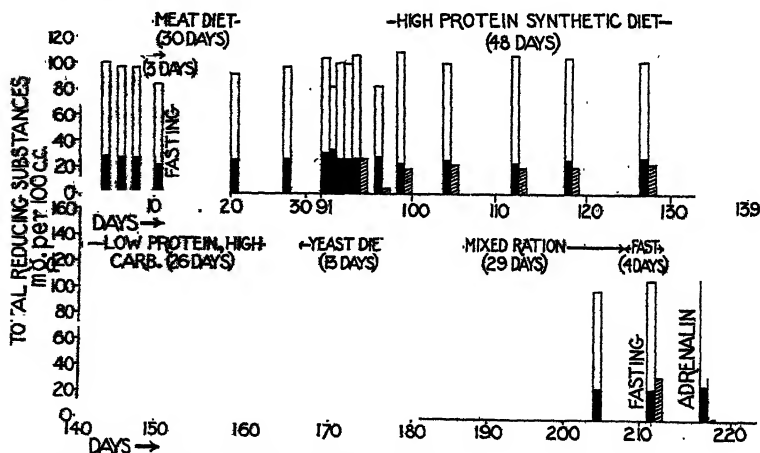


FIG. 2. For the explanation of the symbols, see the legend to Fig. 1.

dogs remained at a relatively constant level. Rarely were there fluctuations of more than 1 or 2 mg. per cent, regardless of the nature of the diet.

Having found from the above dietary studies, in which the successive blood samples were drawn at approximately the same hour each day, that the saccharoid values underwent little or no change from day to day, we investigated the possibility of a diurnal fluctuation. Samples were drawn at 3 p.m. and then at either 12 midnight or 5 a.m. In five such experiments no evidence was obtained that the saccharoids undergo a definite diurnal change.

In contradistinction to the failure of dietary variation to influence the blood saccharoid content, fasting for 3 or 4 days effected a small but definite reduction. In one group of experiments three dogs were fasted for 72 hours and their saccharoids decreased from 29 to 27, from 31 to 26, and from 27 to 22 mg. per cent. In another experiment upon two dogs, bloods drawn after 96 hours of fasting showed saccharoid contents of 22 and 23 mg. per cent, representing a reduction of 5 mg. per cent from the usual saccharoid level of these animals. One dog was fasted in both sets of experiments; on each occasion the saccharoids fell to the apparently basal level of 22 or 23 mg. per cent, in contrast to the consistent figure at other times of 28 or 29 mg. per cent.

In addition to diet and fasting, the influence of insulin and of adrenalin upon the saccharoids was investigated. 20 units of insulin per kilo were injected intracardially into each of three dogs. Determinations were carried out before and approximately 1 hour and 2½ hours after the injection. While insulin markedly reduced the total blood sugar, the saccharoid changes took no definite trend, being somewhat increased in one dog, somewhat decreased in another, and undergoing no variation in the third. A single adrenalin experiment was carried out upon each of the two dogs of our dietary studies. We injected 1 cc. of 1:1000 adrenalin per kilo subcutaneously and withdrew a blood sample after 10 minutes from one dog and after 35 minutes from the other. Notwithstanding the marked increase in the total reducing substances, the saccharoids underwent no deviation from the level previously determined for each dog. This observation that adrenalin has no effect on the saccharoid values in dogs corroborates

a similar result obtained by Folin and Svedberg (1) upon human subjects.

#### SUMMARY

With the Shaffer-Hartmann blood sugar reagent we have found the direct yeast procedure to be the method of choice in the determination of saccharoids. The indirect method through differential filtrate analysis proved less reliable in the determination of saccharoids. Factors in the protein-precipitating solution apparently may influence the reduction of various blood sugar reagents, particularly at high blood sugar levels. When the Shaffer-Hartmann reagent was employed, we obtained low reduction values upon Folin-Wu filtrates at elevated blood sugar levels.

The saccharoid content of the blood is independent of changes in the diet and of diurnal fluctuations, and is relatively constant over long periods of time. Fasting effects a moderate reduction in the blood saccharoid content, possibly to a basal level for the animal in question.

Insulin and adrenalin do not influence the blood saccharoid content.

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## A RAPID PROCEDURE FOR ESTIMATING THE TRYPTOPHANE CONTENT OF CASEIN

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Some 10 years ago, one of us (S.) had occasion to compare the relative tryptophane content of grain curd casein made according to the procedure of Clark *et al.* (1) as such and after treatment to remove the antineuritic vitamin. The treatment was leaching for 1 week with 0.2 per cent acetic acid as recommended by McCollum and coworkers (2), extraction with alcohol and ether, and drying at 140° for 2 hours. It was found that the tryptophane content of the casein was markedly lowered by the alcohol-ether extraction and baking.

The procedure used for the determination of the tryptophane was that of May and Rose (3), except that longer time (7 to 9 days) was employed for maximum color development, as recommended by Holm and Greenbank (4).

Because of the long time required for the maximum color development, attempts were made to shorten the method. In accordance with the suggestion of Komm (5) and of Boyd (6) that mild oxidizing agents furthered the development of the blue color which arises by action of *p*-dimethylaminobenzaldehyde on casein in the presence of relatively strong HCl, a procedure was devised whereby by addition of weak H<sub>2</sub>O<sub>2</sub> the color developed within 30 minutes gave approximately the same comparative results as that developed in 7 days with the Holm and Greenbank modification of the May and Rose procedure; that is, the untreated casein gave much more color than the treated casein. The early work was purely comparative, without reference to the absolute amounts of tryptophane in the various casein samples. Subsequently, as detailed in the present paper, a quantitative



comparison was made of results with the long method and with the short method. Most of the study was carried out on casein obtained from the Will Corporation.

In the long method the tryptophane content was estimated in five different series of experiments with 0.1 gm. samples of the same casein with tryptophane as the standard. In each separate series of determinations nine batches of casein were placed in Erlenmeyer flasks with 100 cc. of the reagents as used by May and Rose, and in nine other flasks were placed 2 mg. of tryptophane in each 100 cc. of the reagent; the eighteen flasks were placed in an incubator set at 37°. Both sets of flasks showed a blue color which increased to a maximum, the 9th day on the average, stayed at a plateau for about 36 hours, and then faded or changed shade. The color of the tryptophane at its maximum was compared with that of the casein at its maximum. The work indicated that the casein in question contained 2.4 per cent tryptophane. Practically the same value was obtained in the five different experiments.

Comparisons were then made between casein in the short method and casein in the May and Rose procedure with color development for 7 to 8 days. The short procedure is as follows: To 99 cc. of 17.5 per cent HCl was added 1 cc. of a 5 per cent solution of *p*-dimethylaminobenzaldehyde in 10 per cent H<sub>2</sub>SO<sub>4</sub>. To each 100 cc. of the acid reagent was added 0.1 gm. of casein. The mixture in each 250 cc. Erlenmeyer flask was brought to 85° and maintained at this temperature for 15 minutes. Then 0.3 cc. of a 0.3 per cent H<sub>2</sub>O<sub>2</sub> solution was added and the contents of the flask were well shaken. The solutions after being cooled in a tap water bath (20-25°) were made to 100 cc. with water. The blue color was compared with that developed in the long procedure at its maximum, 7 to 8 days.

A number of colorimetric determinations were carried out on the same casein by the short procedure. The agreement was excellent. Thus, over a period of 4 weeks, any one of the duplicate series in each day placed at a depth of 20 mm. in the colorimeter checked 20 to 20 ( $\pm 5$  per cent) with the other. Also each day the freshly prepared solution matched with the sample prepared the previous day. The color was found to be stable for 48 hours and then diminished in intensity.

It would seem that the rapid hydrolysis at 85° accelerated by dilute  $H_2O_2$  permits the development of a maximum intensity of color and gives with the casein the same value as the 7 to 8 day color development in the May and Rose procedure; namely, 2.4 per cent tryptophane.

By the short procedure practically the same value was obtained with different samples of casein, a grain curd casein, a Hammarsten casein from Merck and Company, a casein from the Will Corporation, and a casein obtained from Dr. D. B. Jones of the United States Department of Agriculture, a casein which he reports (7) as containing 2.2 per cent tryptophane when determined by a long May and Rose procedure with zein containing a known quantity of tryptophane as a standard. In our work all the caseins were uncorrected for moisture and ash. The drawback in the work is that one batch of casein must be standardized by the long May and Rose procedure as modified by Holm and Greenbank. Then with this casein as a standard, other samples of casein and other proteins can be analyzed by the short procedure, greatly to the advantage of the investigation.

The short procedure has been found very satisfactory by Milone and Sullivan (8) in a comparative study of the tryptophane content of normal and pathological sera.

So far attempts to use tryptophane as a standard in the short method have been unsatisfactory because of the instability of free tryptophane in hot acid solution. Tryptophane dissolved in a mixture of amino acids such as occurs in casein (see Hawk and Bergeim (9)) has been found a better standard than tryptophane alone whether dissolved in dilute alkali, dilute acid, or water. However, even in the amino acid mixture there are indications of more or less loss of reactive tryptophane with the development of a reddish color rather than the blue given by casein.

Since the work on the estimation of tryptophane in casein was finished, Bates (10) reported a modification of the May and Rose procedure whereby, with  $NaNO_2$  as an accelerator, the tryptophane content of proteins can be determined very speedily at room temperature. The Bates method and the short method given in the present paper give for the same casein a blue color development of the same order of magnitude. Bates' longer method with only the accelerator ( $NaNO_2$ ) we have not found

satisfactory with grain curd casein, since a considerable amount of undissolved blue particles was present even after 24 hours incubation at 40°. With tryptophane itself the short method gives a purple-red instead of a blue as with casein. When a blue glass color filter was used to filter out the red, a very low tryptophane value was found for casein, not more than 1.25 per cent, which is much too low.

The comparison of the two short methods and the values found for various proteins with casein as a standard will be discussed in a later paper.

#### SUMMARY

Both by the Holm and Greenbank modification of the May and Rose tryptophane procedure and by a short method the tryptophane content of various caseins has been found to be 2.4 per cent.

The short method involving acceleration of color development by a high temperature and use of dilute  $H_2O_2$  has given consistent and readily reproducible results with various samples of casein.

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## THE NATURE OF VITAMIN A IN COD LIVER OIL\*

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Repeated molecular distillations of fish liver oil, especially Norwegian cod liver oil (1), revealed the fact that vitamin A could be eliminated from the oil in almost quantitative yield at two widely different temperatures. This observation suggested the presence of at least two different forms of this vitamin. Under molecular conditions one form of vitamin A could be distilled at a temperature of approximately 120°, whereas the other form required temperatures ranging from 190–230°. Since the high boiling vitamin could be transformed into the low boiling one by saponification, the assumption was made that vitamin A was present in cod liver oil not only as the free alcohol but also, and to a much greater extent, in an ester form. Recently Reti (2) reported his observations on the state of vitamin A in fish liver oils and stated that all of this vitamin occurs in an ester combination with fatty acids. However, he did not isolate these esters.

While this investigation was in progress, Hamano (3) reported the isolation and identification of vitamin A palmitic acid ester from freshly prepared liver oil of *Steolepis ishinagi*, Hilgendorf. As far as we are aware no attempt has ever been made to determine the state of vitamin A in cod liver oil. This lack of information is probably due to the great difficulties in obtaining a highly potent concentrate of vitamin esters. An attempt to isolate the esters from a commercial grade of oil will result in failure, because of the low concentration of the vitamin.

Since concentrates of high potency can conveniently be prepared by molecular distillation, these were made the basis of an attempt to isolate and identify the natural esters of vitamin A in Norwegian medicinal cod liver oil.

\* Communication No. 607 from the Kodak Research Laboratories.

## EXPERIMENTAL

For the isolation and identification of the ester combinations, use was made of the ability of the conjugated system of double bonds present in the polyene alcohol, vitamin A, to form addition products with maleic anhydride or citraconic anhydride.

*Addition Product of Vitamin A Palmitate with Maleic Anhydride*—Aside from minor differences, the method employed for the isolation of this substance was essentially the same as that given by Hamano (3).

200 gm. of cod liver oil concentrate obtained by molecular distillation, having an apparent vitamin A potency of 325,00 U.S.P. X units per gm. as determined by means of a Hilger vitameter, were dissolved in 200 cc. of dry benzene. To this, 30 gm. of freshly distilled maleic anhydride, dissolved in 300 cc. of benzene, were added and the air in the vessel replaced by nitrogen. After the solution had stood at room temperature for 10 days, the benzene was distilled off and the excess maleic anhydride removed by vacuum distillation. 4 liters of petroleum ether (b.p. 35–65°) were now added. After the solution had stood in a refrigerator for 3 days, a small quantity of fine crystals separated which were filtered off and washed with petroleum ether to remove adhering oily material. The total yield was 1.342 gm. After repeated recrystallizations from acetone, the substance gave a melting point of 219–220° (uncorrected), which is identical with that reported by Hamano (3).

4.466 mg. substance: 11.969 mg. CO<sub>2</sub> and 3.561 mg. H<sub>2</sub>O

C<sub>44</sub>H<sub>64</sub>O<sub>3</sub> (720.5). Calculated. C 73.33, H 8.88

Found. " 73.10, " 8.92

A further identification of the isolated addition compound was made by way of synthesis from a freshly prepared concentrate of the free alcohol, having an apparent (vitameter) potency of 3,500,000 U.S.P. X units per gm., and palmityl chloride in benzene-pyridine solution, according to the method given by Hamano. The compound prepared from this synthetic ester when mixed with the product obtained from cod liver oil concentrate gave no depression of the melting point.

*Palmitic Acid*—A portion of the isolated addition compound was then saponified by refluxing it with 4 N alcoholic potassium

hydroxide for 1 hour. After dilution with water and acidification with hydrochloric acid, a flocculent precipitate formed. This was extracted with petroleum ether and after evaporation of the solvent gave crystals which, after purification over the barium salt, had a melting point of 63°. When mixed with an authentic sample of palmitic acid, no depression in melting point could be observed.

#### SUMMARY

A vitamin A ester has been isolated from Norwegian medicinal cod liver oil in the form of its dimaleic anhydride addition compound and has been identified as vitamin A palmitate. It is present in the oil to an extent of approximately 3 per cent of the total esters.

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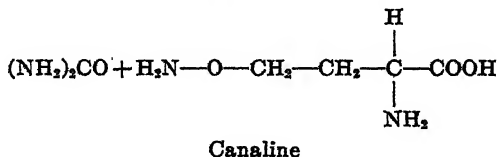
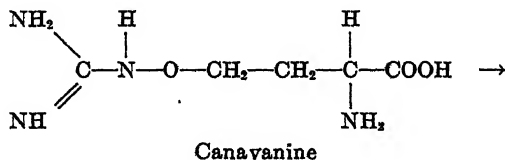
# COMPOUNDS RELATED TO CANALINE AND CANAVANINE\*

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(Received for publication, July 8, 1938)

In 1929 Kitagawa (1-3) and his collaborators isolated from the non-protein fraction of jack bean (*Canavalia ensiformis*) an amino acid, canavanine, which is decomposed by an enzyme present in liver into urea and another amino acid, canaline. He assigned the following formulæ to the two compounds.



These structures were confirmed by means of degradation experiments by Gulland and Morris (4).

The discovery of a naturally occurring ether of hydroxyguanine makes desirable further knowledge of the general properties of this type of compound, and of the related ethers of hydroxylamine, which have received relatively little attention. Experiments have therefore been undertaken to develop convenient

\* This report is from a dissertation submitted by Ernest Borek in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.



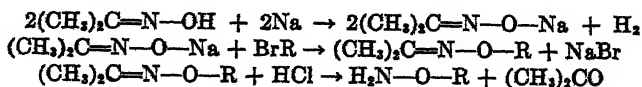
syntheses of representative members of these classes, and, if possible, of canaline.

The dissociation constants of canavanine and canaline have been determined by Tomiyama (5), but in the interpretation of his data this author interchanged the values respectively attributable to the  $\alpha$ -amino and carboxyl groups. He assigned a pK value of 10.3 to the  $-\text{ONH}_2$  group of canaline, thereby indicating it to be more strongly basic than the  $\alpha$ -amino group — an inference which has little plausibility. The dissociation constants of simple derivatives of hydroxylamine and hydroxyguanidine have therefore been determined, and compared with those of canaline and canavanine.

The regeneration of canavanine from canaline has been accomplished by Kitagawa and Takani (6), by the action of methylisourea upon  $\alpha$ -benzoylcanaline. In the endeavor, here described, to effect a total synthesis of canaline, unexpected difficulties were encountered, and all attempts to adapt the several customary syntheses for  $\alpha$ -amino acids have been unsuccessful. While this investigation was in progress, Kitagawa (7) regenerated canaline from the  $\gamma$ -hydroxy- $\alpha$ -aminobutyric acid obtained by the catalytic reduction of canaline. This phase of the present work has been abandoned, but some of the observations made during its unsuccessful progress may be of sufficient interest to record.

$\alpha$ -Substituted hydroxylamines have been prepared by Werner (8, 9) from benzenylamidoxime by an elaborate procedure in which the isolation of intermediate and final products was hampered by the possible formation of isomers. Later, Traube and his collaborators (10) prepared some  $\alpha$ -substituted hydroxylamines by condensing alkyl halides and sulfates with potassium hydroxylamine disulfonate; since the intermediate products are insoluble in organic liquids, this method does not readily lend itself to organic syntheses involving a series of reactions.

The synthesis here described was carried out by condensing the sodium salt of acetoxime with alkyl halides and converting the product by acid hydrolysis into the corresponding  $\alpha$ -substituted hydroxylamine.



The substituted acetoximes can usually be distilled and can be readily used in organic reactions, giving very satisfactory yields. The formation of isomers is excluded, and on acid hydrolysis a single product results.

Hydroxyguanidine has apparently been prepared only once (11), and none of its derivatives was known prior to the discovery of canavanine. Carboxymethoxyguanidine has recently been synthesized, but inadequately characterized, by Kitagawa and Takami (6). In the present work the guanidoxyl derivatives were synthesized by the condensation of  $\alpha$ -substituted hydroxylamines with S-methylisothiourea sulfate (12).

Carboxymethoxyguanidine (guanidoxyacetic acid) was prepared in this way from carboxymethoxylamine (13). Unlike glycoxyamine, creatine, and the benzhydroxamidine derivative of Werner (8) it shows no tendency to undergo ring closure on treatment with mineral acid. Moreover, potentiometric back titration of a solution to which excess hydrochloric acid had been added showed no change in  $pK$  (14).

#### *Titration Curves (Figs. 1 and 2)*

Since  $\alpha$ -substituted hydroxylamines react with quinuhydrone and are readily reduced by hydrogen in the presence of platinum, the glass electrode was used for the potentiometric titrations. All the constants are calculated on the hydrogen scale;  $pK_1$  is the apparent dissociation constant of carboxyl groups, and  $pK_2 = pK_w - pK_B$  that of the basic (aminoxyl and guanidoxyl) groups.

Although the potentiometric titration curve of hydroxylamine has not previously been carried out, its dissociation constant has been calculated from other data. Winkelblech (15) from studies of the conductivity of hydroxylamine hydrochloride solutions reported  $K_B = 7.35 \times 10^{-9}$ ;  $pK = 5.89$ . Ölander (16) obtained its titration curve using colorimetric methods for the pH determination and found  $K_B = 1.07 \times 10^{-8}$ ; whence  $pK = 6.03$ . Berthoud and Eichenberger (17) from colorimetric determinations of the pH of hydroxylamine hydrochloride solutions of different concentrations found  $pK = 6.0$  to 6.1. These are in good agreement with our results (Table I).

$\alpha$ -Substituted hydroxylamines are weaker bases than the parent substance. Carboxymethoxylamine is so weak a base ( $pK_2 =$

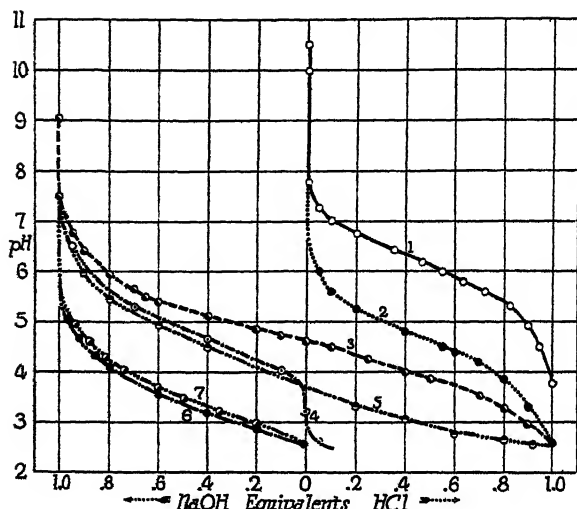


FIG. 1. Titration curves. Curve 1,  $\text{NH}_2\text{OH}$ ; Curve 2,  $\text{NH}_2\text{OCH}_3$ ; Curve 3,  $\text{NH}_2\text{OCH}_2\text{CH}_2\text{CH}_2\text{COOH}$ ; Curve 4,  $\text{NH}_2\text{OCH}_2\text{CH}_2\text{CH}_2\text{COOH}$  in 18 per cent formaldehyde solution; Curve 5,  $\text{NH}_2\text{OCH}_2\text{COOH}$ ; Curve 6,  $\text{NH}_2\text{OCH}_2\text{COOH}$  in 18 per cent formaldehyde solution; Curve 7,  $(\text{CH}_3)_2\text{C}=\text{NOCH}_2\text{COOH}$ .

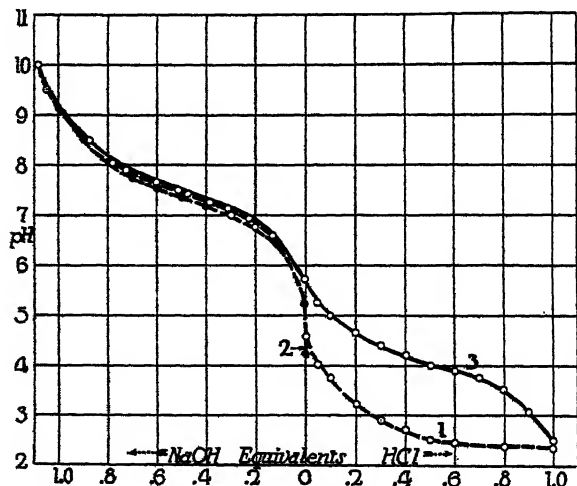


FIG. 2. Titration curves. Curve 1,  $\text{NH}_2\text{C}(\text{NH})\text{NHOCH}_2\text{COOH}$ ; Curve 2,  $(\text{NH}_2\text{C}(\text{NH})\text{NHOCH}_2)_2\text{H}_2\text{SO}_4$ ; Curve 3,  $\text{NH}_2\text{C}(\text{NH})\text{NHOCH}_2\text{CH}_2\text{CH}_2\text{COOH}$ .

4.67) that it crystallizes from solutions containing excess HCl as the semihydrochloride (13). On the other hand,  $\gamma$ -carboxypropoxylamine, in which the basic group is stronger and the carboxyl weaker, crystallizes under the same conditions as the normal hydrochloride.

TABLE I

*Summary of Apparent Dissociation Constants of Some Substances Related to Canaline and Canavanine*

	pK <sub>1</sub>	pK <sub>2</sub>	
		—COOH	—ONH <sub>2</sub> —ONHC(NH)NH <sub>2</sub>
NH <sub>2</sub> OH.....			6.10
NH <sub>2</sub> OCH <sub>3</sub> .....			4.62
NH <sub>2</sub> OCH <sub>2</sub> COOH.....	2.87	4.67	
NH <sub>2</sub> OCH <sub>2</sub> COOH in CH <sub>2</sub> O.....	3.46		
(CH <sub>3</sub> ) <sub>2</sub> C=NOCH <sub>2</sub> COOH.....	3.56		
NH <sub>2</sub> OCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> COOH.....	3.83	5.23	
NH <sub>2</sub> OCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> COOH in CH <sub>2</sub> O.....	4.94		
NH <sub>2</sub> C(NH)NHOCH <sub>3</sub> .....			7.46
NH <sub>2</sub> C(NH)NHOCH <sub>2</sub> COOH.....	2.68		7.51
NH <sub>2</sub> C(NH)NHOCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> COOH.....	4.07		7.59

TABLE II

*Dissociation Constants of Canavanine and Related Compounds*

	pK <sub>1</sub>	pK <sub>2</sub>	pK <sub>3</sub>
Canaline.....	2.40 (COOH)	4.3 (—ONH <sub>2</sub> )	9.20 ( $\alpha$ -NH <sub>2</sub> )
Canavanine ..	2.50 "	7.40 (—ONHC- (NH)NH <sub>2</sub> )	9.25 "
Alanine (20) ..	2.61 "	9.72 ( $\alpha$ -NH <sub>2</sub> )	
Arginine (20) ..	2.24 "	6.9 "	14 (—NHC- (NH)NH <sub>2</sub> )

In formaldehyde solutions the basic character of  $\alpha$ -substituted hydroxylamines disappears; in 18 per cent formaldehyde solution the pK of carboxymethoxylamine on the alkaline side is essentially the same as that of acetone carboxymethoxime in water.

Although guanidine and most of its alkyl derivatives are very

strong bases (18), the relatively weakly basic character of the guanidoxyl group, which contains the strongly electronegative oxygen atom, conforms with the concept involving resonance outlined by Pauling (19).

The pK values for the aminoxyl (4.6 to 5.2) and the guanidoxyl (7.4 to 7.6) groups, shown in Table I, are in good agreement with those reinterpreted from the data of Tomiyama (5) for canaline and canavanine (Table II). For comparison, the values for alanine and arginine, quoted by Clark (20), are included.

#### *Attempted Syntheses of Canaline*

In the first attempts to synthesize canaline, acetone  $\beta$ -bromoethoxime was condensed with sodium phthalimidomalonate ester. The product, on acid hydrolysis, should have yielded canaline. Although sodium bromide was formed during the condensation reaction, glycine was the sole product obtained on acid hydrolysis. This finding is not without parallel. Dunn and Smart (21), in a study of the optimum conditions for the preparation of  $\alpha$ -amino acids by this method, found that, whereas trimethylene bromide and  $\gamma$ -chlorobutyronitrile condense with phthalimidomalonate ester, ethylene bromide,  $\beta$ -chloropropionitrile, and ethyl  $\beta$ -chloropropionate do not do so.

The next method tried involved the bromination and ammonolysis of acetone- $\gamma,\gamma$ -dicarboxy (or  $\gamma,\gamma$ -dicarbethoxy) propoxime. From the bromination of the dicarboxy compound  $(\text{CH}_3)_2\text{C}=\text{NOCH}_2\text{CH}_2\text{CH}(\text{COOH})_2$  there was obtained a very unstable bromo compound containing 2 atoms of bromine, 1 of which is ionizable. This compound decomposes even in the dry state, yielding bromine and hydrogen bromide. In aqueous or ammoniacal solutions it forms an uncrystallizable tar which on acid hydrolysis yields nitric acid and some hydroxylamine. This finding was unexpected, for experiment had shown acetone carboxymethoxime to be unaffected by bromine under the conditions employed.

The bromination of benzoyl- $\gamma,\gamma$ -dicarboxypropoxylamine,  $\text{C}_6\text{H}_5\text{CONHOCH}_2\text{CH}_2\text{CH}(\text{COOH})_2$ , yielded a stable, crystalline bromine derivative containing no ionizable bromine. This substance on decarboxylation yielded a crystalline compound which gave good analyses for benzoyl- $\gamma$ -bromo- $\gamma$ -carboxypropoxylamine,  $\text{C}_6\text{H}_5\text{CONHOCH}_2\text{CH}_2\text{CHBrCOOH}$ , but on treatment with am-

monia broke down into benzamide and an uncrystallizable tar. The original bromination product, upon ammonolysis, yielded a crystalline but unstable product containing 3 atoms of nitrogen, of which 2 could be liberated as ammonia. The crystalline product on warming with 1 equivalent of  $N$  HCl yielded benzamide and an uncrystallizable tar.

Attempts to synthesize canaline by means of the procedures developed by Curtius (22) and by Pyman (23) were likewise unsuccessful. Experimental details of these attempts are not here reported.

#### EXPERIMENTAL

*N-Benzoylcarboxymethoxylamine*—This compound was prepared by Werner (8) and by Kitagawa and Takani (6) by methods different from that here described. To 1 gm. of carboxymethoxylamine semihydrochloride in 20 cc. of 2  $N$  NaOH, 1.5 gm. of benzoyl chloride were added in five portions with vigorous stirring. After about 30 minutes the solution was acidified to Congo red with 3  $N$  HCl; the product was extracted with ether, and dissolved in 5 cc. of hot ethyl acetate. On the addition of 35 cc. of carbon tetrachloride 0.9 gm. (53 per cent of the theoretical amount) of benzoylcarboxymethoxylamine crystallized out free of benzoic acid, which under these conditions remains in solution. After two recrystallizations the melting point was constant at  $123^{\circ}$  (Werner (8) reports a melting point of  $135-138^{\circ}$ ; Kitagawa and Takani (6)  $139-140^{\circ}$ ).

*Analysis*— $C_8H_9O_4N$ . Calculated. C 55.4, H 4.6, N 7.2  
Found. " 55.4, " 4.7, " 7.3

*Acetone  $\beta$ -Bromoethoxime*—To a cooled solution of 125 gm. of sodium in 3 liters of absolute alcohol, 400 gm. of acetoxime were added. The alcohol was evaporated under diminished pressure, and the residue dried first on the steam bath and finally under 1 mm. pressure in an oil bath at  $110^{\circ}$  for 1 hour. The crystalline mass was vigorously shaken with 1.5 liters of toluene on a shaking machine until it became a thin paste. It was then added in five portions during an hour to 4 kilos of gently boiling ethylene dibromide, with mechanical stirring. The mixture was gently boiled until neutral to litmus (about 2.5 hours). During the reac-

tion about 23 gm. of vinyl bromide were evolved and collected in a trap cooled with solid  $\text{CO}_2$ . The sodium bromide was removed and washed with toluene. The combined filtrates were fractionated through an eight plate bubbler column (24) at 30 mm., and the fraction distilling up to  $35^\circ$  (bath up to  $70^\circ$ ) was discarded. The residue was dissolved in 600 cc. of ether and washed three times with 150 cc. portions of water. The dried ethereal residue was distilled under 1 mm. pressure, the fraction boiling at  $36\text{--}45^\circ$  (bath temperature  $50\text{--}65^\circ$ ) being collected. The yield was 200 gm. (20 per cent of the theoretical amount).

*Analysis*— $\text{C}_8\text{H}_{10}\text{ONBr}$ . Calculated. C 33.3, H 5.6, N 7.8, Br 44.4  
Found. " 32.8, " 5.3, " 7.7, " 43.6

*Acetone  $\gamma,\gamma$ -Dicarbethoxypropoxime*—To a cooled solution of 8.5 gm. of sodium in 200 cc. of freshly distilled absolute ethyl alcohol, 90 gm. (50 per cent excess) of ethyl malonate were added, followed by 67 gm. of acetone  $\beta$ -bromoethoxime in four portions. The whole was gently boiled on the steam bath, with stirring, for 1.5 hours. The mixture was cooled, diluted with 300 cc. of water, and extracted with 1250 cc. of ether in five portions. The ethereal extract was distilled under reduced pressure; b.p.  $120\text{--}128^\circ$  at 2 mm. The yield was 70 gm. (72 per cent of the theoretical amount).

*Analysis*— $\text{C}_{12}\text{H}_{21}\text{O}_5\text{N}$ . Calculated. C 55.6, H 8.1, N 5.4  
Found. " 55.2, " 8.0, " 5.3

*Acetone  $\gamma,\gamma$ -Dicarboxypropoxime*—To 9.7 gm. of the above ester in 15 cc. of ethyl alcohol 40 cc. of 5 N sodium hydroxide were added. After being boiled for 1 hour the mixture was cooled to  $0^\circ$  and acidified to Congo red with 6 N sulfuric acid, saturated with sodium chloride, and extracted with 400 cc. of ether in five portions. The ethereal extract, on evaporation, crystallized in long needles. These were recrystallized from a mixture of ethyl acetate and carbon tetrachloride. The yield was 6.8 gm. (89 per cent of the theoretical amount); m.p.  $113^\circ$ . It was soluble in water, alcohol, and ether; insoluble in chloroform and carbon tetrachloride.

*Analysis*— $\text{C}_8\text{H}_{11}\text{O}_5\text{N}$ . Calculated. C 47.3, H 6.4, N 6.9  
Found. " 47.3, " 6.3, " 6.6

*$\gamma, \gamma$ -Dicarbethoxypropoxylamine Hydrochloride*—To a solution of 27 gm. of acetone  $\gamma, \gamma$ -dicarbethoxypropoxime in 700 cc. of absolute ethyl alcohol, 50 cc. of concentrated hydrochloric acid were added and the mixture was slowly distilled through a long Vigreux column, the volume being kept constant by the addition of alcohol. After 5 hours the solution was concentrated under diminished pressure to a thick syrup; this was dissolved in 20 cc. of absolute alcohol and 400 cc. of ether were added. The clear solution, in the ice box, deposited 10 gm. of the ester hydrochloride. The mother liquor, concentrated to a syrup under diminished pressure, and distilled as before with 700 cc. of alcohol and 50 cc. of concentrated hydrochloric acid, yielded on further distillation 6.5 gm. of crystals. Two repetitions of the process yielded crops of 5.0 and 3.5 gm. The total yield of 25 gm. represents 92 per cent of the theoretical amount. The product after recrystallization from alcohol-ether solution melted at 103°.

*Analysis*— $C_8H_{13}O_5NCl$ . Calculated. C 42.3, H 7.0, N 5.5, Cl 13.9  
Found. " 42.1, " 6.8, " 5.8, " 13.7

*Benzoyl- $\gamma, \gamma$ -Dicarboxypropoxylamine*—To 5.5 gm. of the above hydrochloride in 80 cc. of saturated sodium bicarbonate solution, 4.4 cc. of benzoyl chloride were added in four portions at 10 minute intervals, with vigorous stirring. After the odor of benzoyl chloride was no longer detectable, the mixture was extracted with 125 cc. of ether. The ether solution was evaporated; 42 cc. of *N* potassium hydroxide were added to the residue and the mixture refluxed for 1 hour. The solution was concentrated under diminished pressure to a heavy syrup which was stirred with 400 cc. of absolute alcohol. The insoluble dipotassium salt was filtered off, dissolved in 35 cc. of water, acidified to Congo red with hydrochloric acid, and extracted with 100 cc. of ethyl acetate in three portions. The combined extracts were dried in a vacuum desiccator. The crystalline mass was taken up in 150 cc. of boiling ethyl acetate and carbon tetrachloride (75 cc.) was added to incipient precipitation. After 6 hours in the ice box 100 cc. more of carbon tetrachloride were added to complete crystallization. The yield was 4.4 gm. (76 per cent of the theoretical amount). After three such crystallizations, the melting point was 150°.



*Analysis*— $C_{12}H_{15}O_5N$

Calculated. C 53.9, H 4.9, N 5.2, neutralization equivalent 133.5

Found. " 53.6, " 4.7, " 5.2, " " 132

*Benzoyl- $\gamma$ -Carboxypropoxylamine*—3.1 gm. of benzoyl- $\gamma, \gamma$ -dicarboxypropoxylamine were heated at 155–160° for 25 minutes. When cold, the crystalline mass was dissolved in 30 cc. of boiling ethyl acetate and 100 cc. of carbon tetrachloride were slowly added to the solution. The yield was 2.4 gm. (93 per cent of the theoretical amount). After two recrystallizations the melting point was 112°.

*Analysis*— $C_{11}H_{13}O_4N$ . Calculated. C 59.2, H 5.8, N 6.3

Found. " 58.8, " 6.2, " 6.2

*$\gamma$ -Carboxypropoxylamine Hydrochloride*—A solution of 500 mg. of the above benzoyl derivative in 30 cc. of 3 N HCl was boiled for 1.5 hours, cooled, filtered from benzoic acid, and evaporated to dryness under diminished pressure. The crystalline residue was taken up in 4 cc. of 98 per cent alcohol and 50 cc. of ether were added. The needles after recrystallization from alcohol-ether melted at 142°.

*Analysis*— $C_4H_9O_2NCl$ . Calculated. C 31.0, H 6.5, N 9.0

Found. " 31.1, " 6.7, " 8.9

*$\gamma$ -Carboxypropoxylamine hydrochloride* was also prepared by the decarboxylation and subsequent hydrolysis of acetone  $\gamma, \gamma$ -dicarboxypropoxime. 4.6 gm. of this substance were heated at 130° for 15 minutes. The resulting syrup was dissolved in 40 cc. of 3 N HCl. The solution was gently boiled for 2 hours under a long Vigreux column so as to remove acetone, then evaporated to dryness under diminished pressure. The crystalline residue was dissolved in 10 cc. of cold 95 per cent alcohol; 150 cc. of ether were added, and the solution was placed in the ice box overnight. The yield was 2.6 gm. On repetition of the process with the mother liquor a second crop was obtained. The total yield was 3.3 gm. (89 per cent of the theoretical amount); m.p. 142°.

*Methoxyguanidine Sulfate*—To 46.4 gm. of methylisothioureia sulfate in 300 cc. of water 20 gm. of  $\alpha$ -methylhydroxylamine in 45 cc. of water were added slowly, with stirring, during 2 hours,

the temperature being held at 70°. The solution was then boiled for 1.5 hours under a reflux to expel residual methyl mercaptan. It was then concentrated to about 100 cc. under diminished pressure; 50 cc. of 95 per cent alcohol were added and the color removed with charcoal. To the filtrate 400 cc. of 95 per cent alcohol were added, and the cloudy solution was placed in the ice box. At the end of 2 hours a copious amount of crystals had been deposited; 300 cc. of 95 per cent alcohol were added, and the mixture was kept in the ice box overnight. The crystals were filtered off and washed with absolute alcohol. 30 gm. were obtained. The mother liquor, after concentration to 25 cc., yielded 10 gm. more by precipitation with alcohol. The yield was 87 per cent of the theoretical amount. After three crystallizations from water-alcohol solution the melting point was 145–146°.

*Analysis*— $C_4H_{16}O_8N_6S$ . Calculated. C 17.4, H 5.8, N 30.4,  $SO_4$  34.8  
 Found. " 17.5, " 6.2, " 30.1, " 34.8  
 " 17.3, " 6.4

The nitrogen was determined by macro-Kjeldahl procedure after reduction with zinc and hydrochloric acid.

*Carboxymethoxyguanidine*—To a chilled solution of 5.5 gm. of carboxymethoxylamine semihydrochloride in 300 cc. of 0.2 N  $H_2SO_4$  a suspension of silver oxide, prepared from 4.25 gm. of  $AgNO_3$ , was slowly added with stirring. The centrifuged solution and washings were saturated with  $H_2S$ . The colloidal precipitate was coagulated by chilling in solid carbon dioxide and filtered off after thawing. 14 gm. of recrystallized  $Ba(OH)_2 \cdot 8H_2O$  were added to the solution, followed by 7.5 gm. of methylisothiurea sulfate. The mixture was stirred at 85–90° for 3 hours, cooled, and centrifuged. The supernatant and washings were freed of barium and concentrated to 50 cc. under reduced pressure. The crystals that appeared were filtered off, and a second crop secured from the filtrate by adding 250 cc. of 1:1 ether-alcohol solution, seeding, and chilling. Total yield, 4.2 gm. (63 per cent of the theoretical amount). After three recrystallizations the melting point was 195°.

*Analysis*— $C_2H_7O_2N_3$ . Calculated. C 27.1, H 5.3, N 31.6  
 Found. " 27.0, " 5.3, " 31.2

A solution of 0.8 gm. of carboxymethoxyguanidine in 3 cc. of 21 per cent HCl was evaporated on the steam bath to a syrup. This crystallized in a vacuum desiccator over  $P_2O_5$  and KOH, and was further dried for 2 hours at  $100^\circ$  over  $P_2O_5$  *in vacuo*. The melting point was  $110-111^\circ$ .

*Analysis*— $C_2H_5O_3N_2Cl$ . Calculated. C 21.2, H 4.7, Cl 20.9  
Found. " 21.4, " 4.5, " 21.0

On treatment in concentrated aqueous solution with pyridine the original product crystallized. It gave no depression of melting point when mixed with untreated material.

*$\gamma$ -Carboxypropoxyguanidine*—A chilled solution of 2.5 gm. of  $\gamma$ -carboxypropoxylamine hydrochloride in 50 cc. of 0.1 N sulfuric acid was treated as above described with silver oxide, made alkaline to litmus with barium hydroxide, and heated with 3 gm. of methylisothiurea sulfate. After removal of barium the solution was evaporated to dryness under diminished pressure. The residual syrup was dissolved in 10 cc. of water; 80 cc. of alcohol and then 100 cc. of ether were added, and the cloudy solution placed in the ice box. After 6 hours 100 cc. more ether were added to complete the crystallization. The yield was 1.6 gm. (60 per cent of the theoretical amount). After three recrystallizations from aqueous alcohol it melted at  $205^\circ$ .

*Analysis*— $C_5H_{11}O_3N_3$ . Calculated. C 37.3, H 6.8, N 26.1  
Found. " 37.2, " 6.7, " 25.7

*Stability of Acetone Carboxymethoxime to Bromine*—To a solution of 0.5 gm. of acetone carboxymethoxime in 5 cc. of alcohol-free ether 0.6 gm. (0.2 cc.) of bromine was added. The solution was allowed to remain at room temperature ( $24^\circ$ ) for 2 hours. The ether and most of the bromine were evaporated under diminished pressure, the residue was dissolved in 15 cc. of ether, and the solution again evaporated. The brown syrup was dissolved in 2 cc. of ethyl acetate and carbon tetrachloride (about 4 cc.) was added until it became cloudy. The crystalline product obtained on inoculation and chilling was recrystallized as above. It did not depress the melting point of the original substance.

*Bromination of Acetone  $\gamma,\gamma$ -Dicarboxypropoxime*—To 8.5 gm.

of acetone  $\gamma, \gamma$ -dicarboxypropoxime in 100 cc. of dry, peroxide-free ether, 22 cc. of a solution of bromine in carbon tetrachloride containing 30 gm. per 100 cc. were added in five portions during 30 minutes. All the bromine was taken up, and an oil precipitated which readily crystallized when scratched. The crystalline mass was ground and washed with 250 cc. of ether. The yield was 13 gm. The product was dried *in vacuo* at  $70^\circ$  for analysis.

*Analysis*— $C_8H_{13}O_5NBr_2$

Calculated. C 26.5, H 3.5, N 3.9, Br 44.1, Br- 22.1

Found. " 27.2, " 3.5, " 4.1, " 41.8, " 22.8

" 27.1, " 3.6, " 4.3

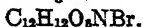
The crystalline compound is very unstable; bromine and HBr were liberated in a few days even from the dry product. In aqueous solution it evolved carbon dioxide at  $25^\circ$ , and on concentration under diminished pressure yielded a purple tarry mass. An ether extract of a decarboxylated aqueous solution, on evaporation, also yielded only a tar.

The brominated acid was dissolved in 20 times its weight of cold concentrated ammonia and allowed to remain at  $20^\circ$  for 50 hours. On application of customary isolation procedures, there was obtained an amorphous, brown product, soluble in water but insoluble in alcohol. The alcohol solution when evaporated yielded ammonium nitrate, and, in runs in which the product after ammonolysis was subjected to acid hydrolysis, some hydroxylamine.

*Bromination of Benzoyl- $\gamma, \gamma$ -Dicarboxypropoxylamine*—After it had been established that benzoylcarboxymethoxylamine can be recovered unchanged after treatment with bromine, a solution of 2 gm. of benzoyl- $\gamma, \gamma$ -dicarboxypropoxylamine in 150 cc. of ethyl acetate was treated at  $25^\circ$  with 1.4 gm. of bromine in 5 cc. of carbon tetrachloride in two portions. Decolorization was complete in about 20 minutes. The solution was evaporated to dryness under diminished pressure, the crystalline product was dissolved in the least amount of boiling ethyl acetate (about 75 cc.), and after the addition of 150 cc. of carbon tetrachloride it was placed in the ice box. The yield was 2.3 gm. (89 per cent of the theoretical amount). After three recrystallizations the product melted at  $105^\circ$  with evolution of  $CO_2$ . Since the compound is

unstable, it was dried at room temperature, and apparently contained solvent of crystallization.

*Analysis—*



Calculated. C 41.6, H 3.5, N 4.1, Br 23.1, neutralization equivalent 173



Calculated. C 44.2, H 4.6, N 3.2, Br 18.4, neutralization equivalent 217

Found. " 45.0, " 5.0, " 3.2, " 17.2 " " 206

" 45.5, " 4.6, " 3.3

On treatment with ammonia it yielded a crystalline product which separated on the addition of alcohol.

*Analysis—*

$\text{C}_{12}\text{H}_{12}\text{O}_4\text{N} \cdot 2\text{NH}_3$ . Calculated. C 49.5, H 6.5, N 14.4,  $\text{NH}_3$  11.7

Found. " 48.9, " 5.7, " 13.7, " 9.2

When an aqueous solution of this substance was allowed to stand overnight, an uncrystallizable tar was precipitated on adding alcohol. When it was boiled for 5 minutes with 1 equivalent of N HCl, benzamide was produced almost quantitatively, but no other product could be isolated.

*Benzoyl- $\gamma$ -Bromo- $\gamma$ -Carboxypropoxylamine*—When heated at  $110^\circ$ , the above bromodicarboxylic acid gave off carbon dioxide and hydrogen bromide; at  $105^\circ$  only decarboxylation occurred. 1.5 gm. was held at  $105^\circ \pm 1^\circ$  for 15 minutes; the product, which crystallized on cooling, was recrystallized from ethyl acetate-carbon tetrachloride. The yield was 1.1 gm.; m.p.  $149^\circ$ .

*Analysis*— $\text{C}_{11}\text{H}_{12}\text{O}_4\text{NBr}$ . Calculated. C 43.7, H 4.0, N 4.6, Br 26.4

Found. " 44.0, " 4.0, " 4.6, " 26.3

On treatment with excess cold, concentrated ammonia this compound yielded benzamide and an uncrystallizable tarry product.

*Attempted Condensation of Acetone  $\beta$ -Bromoethoxime with Ethyl Sodium Phthalimidomalonate*—Benzene, toluene, xylene, phenetole, and cymene were tried as diluents for the condensation. The reaction in phenetole will serve as an example of the method. 15.2 gm. of acetone  $\beta$ -bromoethoxime, 18.6 gm. of ethyl sodium phthalimidomalonate, and 45 cc. of phenetole were heated at  $155$ – $160^\circ$  for 1.25 hours with stirring. When cold, the brown mixture

was treated with ether and filtered. The ether was evaporated and the phenetole removed by distillation at 1 mm. The brown, glassy residue (16.5 gm.) was dissolved in 30 cc. of glacial acetic acid and 40 cc. of 12 N  $\text{H}_2\text{SO}_4$ , and boiled under a reflux for 4 hours. 80 cc. of water were added and the mixture was extracted with 400 cc. of ether in five portions to remove all the phthalic acid. The aqueous portion was diluted with water, freed of sulfate, and concentrated under diminished pressure. The syrup yielded 3.25 gm. of crystals. To 520 mg. of these, saturated rufanic acid solution was added; only 50 mg. of precipitate were obtained. The excess rufanic acid was removed from the filtrate with  $\text{Ba}(\text{OH})_2$  and the excess barium removed with  $\text{H}_2\text{SO}_4$ . The solution was concentrated under diminished pressure and treated with excess absolute alcohol, when 400 mg. of crystals, which were found to be pure glycine, were obtained.

#### *Titration Curves*

0.05 M solutions were titrated at  $23^\circ \pm 2^\circ$  with N sodium hydroxide and with N hydrochloric acid. The dilution was therefore not greater than 5 per cent. The pH measurements were made with a glass electrode (25) which had been calibrated with standard buffers. The measurements are reliable to  $\pm 0.02$  pH unit. Each constant reported is the average of five points on curves corrected for water blanks.

In the formaldehyde titrations the solutions were 0.05 M in 18 per cent formaldehyde solution.

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#### SUMMARY

The preparation of several carboxylated ethers of hydroxylamine and hydroxyguanidine is described. The apparent dissociation constants of these substances are in good agreement with those of the corresponding groups in canaline and canavanine.

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## THE PREPARATION OF FATTY ACIDS CONTAINING DEUTERIUM\*

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In this paper we describe two methods for the preparation of deuterofatty acids, both of which are based on the fact that carbon-bound hydrogen may under exceptional conditions become labile and exchange with the deuterium of solvents. The first method, namely the treatment of ordinary fatty acids with hot concentrated  $D_2SO_4$ , has been reported in a preliminary note (1). This method is based on the finding of Ingold, Raisin, and Wilson (2) that some hydrocarbons exchange their hydrogen with that of  $D_2SO_4$ . Saturated fatty acids are very resistant to hot sulfuric acid, and the loss of organic material after such treatment is very small. The deuterium thus introduced is stably bound; it is not removed from the fatty acids by treatment with boiling dilute 20 per cent sulfuric acid or with boiling 7 per cent aqueous alcoholic KOH. This was to be expected, as all the hydrogen atoms in fatty acids, except that of the carboxyl group, are known to be stable under such conditions.<sup>1</sup>

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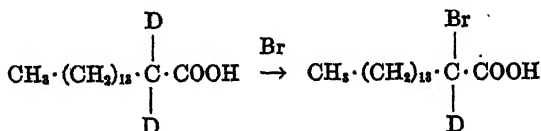
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<sup>1</sup> The hydrogen of fatty acids is also stable in esters. Tributyrin was saponified by refluxing with 7 per cent  $D_2O$  containing 7 per cent NaOH, and the sodium butyrate isolated contained no deuterium. This experiment rules out the possibility that hydrolysis of the tributyrin was accom-

panied by enolization of the group  $R-CH_2-C \begin{smallmatrix} \nearrow OH \\ \searrow O \end{smallmatrix}$



Closer investigation has now shown that by this method less D than is equivalent to 2 deuterium atoms is introduced into the fatty acid molecule. In the cases of palmitic acid it has been shown that the deuterium atoms are attached to the  $\alpha$ -carbon atom, since the conversion into the  $\alpha$ -bromopalmitic acid leads to a loss of exactly half of the total deuterium.



The amount of deuterium which can have been introduced at other carbon atoms must have been negligible.

The method is limited to the preparation of  $\alpha$ -deuterofatty acids, which may prove to be of interest for some biological investigations. It is not applicable to the preparation of unsaturated deuterio acids, since double bonds are attacked by concentrated sulfuric acid.

The second method is based on the labilization of carbon-bound hydrogen by active platinum at high temperatures. Horrex and Polanyi (3) have shown that deuterium is introduced into cyclohexane and isopentane by heating them with heavy water in the presence of active platinum. When the higher fatty acids are shaken with active platinum and  $\text{D}_2\text{O}$  at  $120^\circ$  for several days, only a very small amount of deuterium is introduced into the compounds. Large amounts, however, are introduced on the addition of alkali in quantities insufficient for neutralization of the acid. It may be that in the absence of alkali the orientation of the fatty acid at the oil-water interface is such that only the carboxyl group and the adjacent carbon atoms are in contact with the water. On the addition of alkali part of the acid goes into solution as soap, and an equilibrium exists between the dissolved and the undissolved phase. This may permit the hydrogen atoms of the chain to react. The effect of alkali might also be due to basic catalysis.

The method has the advantage that the deuterium atoms are introduced at many, and perhaps all, of the carbon atoms. By varying the deuterium concentration of the water it is possible to prepare acids of any desired deuterium content. No loss of fatty

acids is involved, and the heavy water, the deuterium concentration of which is correspondingly decreased, can be quantitatively recovered.

#### EXPERIMENTAL

##### *Exchanges with $D_2SO_4$*

*Exchange of Deuterium between Palmitic Acid and  $D_2SO_4$* —Concentrated  $D_2SO_4$ , 100 per cent with respect to sulfuric acid, and containing 95 atom per cent of deuterium was prepared by dissolving 1 mole of  $SO_3$  in 1 mole of 95 per cent  $D_2O$ . 1 gm. of palmitic acid and 1.85 gm. of the 100 per cent  $D_2SO_4$  were weighed into a test-tube, and 0.186 cc. of 95 per cent  $D_2O$  added to bring the sulfuric acid concentration to 90 per cent. The neck of the tube was drawn to a long capillary and the tube placed in an oven at 98–100° for 50 hours. The tube was opened and the dark amber contents precipitated with water, and extracted with ether. The ethereal solution was extracted with alcoholic KOH, and the alkaline solution acidified with dilute  $H_2SO_4$  and extracted again with ether. By this process the labile deuterium of the carboxyl group was completely replaced by ordinary hydrogen. After removal of the well dried ether the acid was recrystallized from aqueous acetone, yielding 913 mg. of palmitic acid, m.p. 62.5°, containing 4.32 atom per cent of deuterium. Assuming that D and H atoms have the same affinity for the carbon atoms, this corresponds to an exchange of 1.38 H atoms per molecule.

Similar tests on 1 gm. quantities of palmitic acid with 95 and 98 per cent  $D_2SO_4$  gave 764 and 593 mg. of acid containing 5.04 and 4.62 atom per cent of deuterium respectively (corresponding to 1.79 and 1.48 atoms of deuterium per molecule). An experiment with 90 per cent  $D_2SO_4$  at 130° yielded 933 mg. of palmitic acid containing 4.6 atom per cent of deuterium (1.47 atoms of deuterium per molecule).

*Preparation of  $\alpha$ -Bromodeuteropalmitic Acid*—1.5 gm. of deuteropalmitic acid, prepared by the exchange with  $D_2SO_4$  and containing 4.65 atom per cent of deuterium, were ground with 0.12 gm. of red phosphorus and transferred to a bromination flask fitted with a capillary dropping funnel and attached to a condenser by means of a ground glass joint. The mixture was treated dropwise with 1.5 cc. of bromine over a period of 20 minutes, and

then heated on a steam bath for 1.5 hours. The contents were poured into dilute  $\text{Na}_2\text{SO}_3$  solution, the precipitate taken up with ether, the ethereal solution washed with water and dried, and the acid recrystallized from light petroleum. Yield, 1.34 gm., m.p. 53–54°. The deuterium content was 2.48 atom per cent. Assuming that all the deuterium in the original acid was on the  $\alpha$ -carbon atom, half should have been removed by bromination. The calculated value for the  $\alpha$ -bromo acid should then be 2.40 atom per cent deuterium.

*Exchanges with  $\text{D}_2\text{O}$  in Presence of Platinum*

Before the saturated fatty acids were subjected to the exchange reaction, they were purified by recrystallization once from hot concentrated  $\text{H}_2\text{SO}_4$ , and then, with the exceptions of caprylic and capric acids, from aqueous acetone. The latter two acids were redistilled under 15 mm. pressure.

*Preparation of Deuterolauric Acid*—750 mg. of platinum oxide in 7.5 cc. of 99.5 per cent  $\text{D}_2\text{O}$  in a long, narrow necked, round bottomed flask are reduced with deuterium gas, 7.5 gm. of lauric acid, m.p. 45.2°, and 100 mg. of KOH are added, and the flask, after being cooled with solid  $\text{CO}_2$ , is evacuated, sealed, and shaken for 6 days at 130–135°. After cooling the flask is opened, the heavy water sublimed off *in vacuo*, the residue acidified with  $\text{P}_2\text{O}_5$ , and the acid extracted with ether. To replace the labile deuterium of the carboxyl group with hydrogen the ethereal solution is extracted with dilute alkali and the acid again extracted after acidification. On recrystallization from a large volume of aqueous acetone, 7.2 gm. of lauric acid, m.p. 45.2°, containing 28.7 atom per cent deuterium are obtained. If complete equilibrium had been attained (*e.g.* equal deuterium concentration with water and the carbon-bound hydrogen atoms), the deuterium content in the isolated fatty acid would have been 48.1 atom per cent.

The deutero acids listed in Table I were prepared in the same way. Column 2 gives the deuterium content of the fatty acids after isolation, and Column 3 the theoretical deuterium content which would be obtained when all the carbon-bound hydrogen atoms are in equilibrium with those of the heavy water with which they are treated. Column 4 gives the ratio of the values in Columns 2 and 3. This is the per cent exchange in each ex-

periment with reference to the calculated equilibrium value; for example, the deuterostearic acid reached 49.2 per cent of the maximum deuterium content.

The deuterium content of the fatty acids is dependent not only upon the concentration of deuterium in the heavy water, but also upon the amount of catalyst (see Series C), and upon the duration of treatment (see Series B). The exchange is a

TABLE I  
*Preparation of Deuterofatty Acids*

Acid		Deuterium content of acid	Deuterium content calculated for equilibrium	Maximal exchange attained
(1)		(2)	(3)	(4)
Series A. 750 mg. $\text{PtO}_2 \cdot \text{H}_2\text{O}$ , 100 mg. KOH in 7.5 cc. of 99% heavy water at 130° for 6 days				
	gm.	atom per cent	atom per cent	per cent
Stearic.....	7.5	22.4 $\pm$ 0.2	45.5	49.2
Palmitic.....	5.0	21.9 $\pm$ 0.4	55.5	39.5
Lauric.....	7.5	28.7 $\pm$ 0.4	46.2	62.1
Series B. 1.5 gm. $\text{PtO}_2 \cdot \text{H}_2\text{O}$ , 100 mg. KOH in 15 cc. of 55% heavy water at 130° for 12 days				
Myristic.....	7.5	29.8 $\pm$ 0.3	34.0	87.5
Capric.....	7.5	13.9 $\pm$ 0.3	34.3	40.6
Caprylic.....	7.5	25.1 $\pm$ 0.4	34.4	73.0
Series C. 100 mg. $\text{PtO}_2 \cdot \text{H}_2\text{O}$ , 100 mg. KOH in 10 cc. of 55% heavy water at 130° for 6 days				
Myristic.....	7.5	4.40 $\pm$ 0.1	29.0	15.2
Capric.....	7.5	1.49 $\pm$ 0.1	29.5	5.1
Caprylic.....	7.5	5.20 $\pm$ 0.1	29.6	17.6

rather slow process, and even after 12 days equilibrium is not reached.

In some cases the catalyst coagulated during the treatment and was consequently not well distributed through the reaction mixture. In such cases the final deuterium content of the fatty acids was low, as for instance with the capric acid of Series C in Table I. In a 6 day experiment with myristic acid, not shown in Table I, no deuterium was introduced, owing to coagulation of the catalyst.

*Stability of Deuterium in Palmitic Acid Obtained by Treatment with Platinum and Heavy Water*—500 mg. of palmitic acid containing  $5.49 \pm 0.1$  atom per cent deuterium were refluxed for 48 hours with 20 cc. of ethyl alcohol, 18 cc. of  $H_2O$ , and 4 cc. of concentrated  $H_2SO_4$ . After cooling, the contents were extracted with ether, the ether distilled off, and the residue refluxed for 3 hours with 80 per cent methyl alcohol containing 7 per cent KOH. The solution was acidified, extracted with ether, and the solid residue from the ethereal extract recrystallized from aqueous acetone. The resulting palmitic acid had a melting point of  $62.5^\circ$  and contained  $5.46 \pm 0.1$  atom per cent deuterium. The substance had thus not lost any deuterium during the treatment with acid and alkali.

#### SUMMARY

1. Two methods are described for the preparation of fatty acids containing stably bound deuterium.

2. At elevated temperatures fatty acids exchange hydrogen with the deuterium of  $D_2SO_4$  in which they are dissolved. The exchange occurs only at the  $\alpha$ -carbon atom.

3. Saturated fatty acids exchange a large number, probably all, of their hydrogen atoms with the deuterium of  $D_2O$  when heated with  $D_2O$  in the presence of alkali and active platinum.

4. The deuterium thus introduced is not removed by treatment of the acids with alkali or mineral acids at elevated temperature.

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## SULFHYDRYL GROUPS IN PROTEINS

### I. EGG ALBUMIN IN SOLUTIONS OF UREA, GUANIDINE, AND THEIR DERIVATIVES

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Proteins may contain several actual or potential sulfhydryl groups. The importance of these groups for cellular respiration had been recognized early by deRey Pailhade and has been since extensively investigated by Hopkins and his coworkers (11, 13, 14). Such groups appear to be of importance not alone for purely oxidation-reduction mechanisms but for the enhanced functioning of certain hydrolytic biocatalysts such as urease (10, 28), papain (4, 5), kathepsin (19), and cerebrosidase (33). On the other hand, the presence of intact disulfide linkages appears to be necessary to the physiological behavior of other active principles such as insulin (31, 35), the appearance of free —SH groups coinciding with loss in activity.

Proteins, other than the biocatalysts alluded to, such as hemoglobin, myosin, and the lens proteins, may contain in the native state free —SH groups, as the experiments of Todrick and Walker (34), Anson and Mirsky (1), and Mirsky (20) have demonstrated. The number of these free groups in these and many other proteins is increased by treatment of the protein with denaturing agents (20). Methods for estimating the concentration of sulfhydryl groups in native and denatured proteins have been devised by Todrick and Walker (34) and by Mirsky and Anson (21). These have been applied exclusively to heat-denatured or trichloroacetic acid-precipitated proteins which were subsequently investigated in heterogeneous mixtures with various reagents. The method of Todrick and Walker consisted in heating the protein suspension with varying quantities of the oxidation-reduction dye, 2,6-

dichlorophenol indophenol. This dye reacts slowly with protein sulfhydryl at ordinary temperatures. Its reaction with cysteine is not stoichiometric. Kuhn and Desnuelle have introduced the use of porphyrindin for the estimation of protein sulfhydryl groups (15). This dye is a powerful oxidizing agent, reacts rapidly and stoichiometrically with cysteine and with protein —SH groups in the cold, and possesses an  $E_0 = +0.57$  volt at pH 7.0 in comparison with an  $E'_0$  for the indophenol dye of  $+0.22$  volt, at the same pH. The porphyrindin dye is a deep blue in the oxidized state and slightly yellow in the reduced condition. Such a molecule possesses considerable advantages in the present connection and opens many possibilities in other fields as an intermediate of high potential.

It was thought of interest to extend the use of this dye to the estimation of sulfhydryl groups of native and denatured proteins. A remarkable method of altering many proteins is to dissolve them in urea or other amide solutions, whereby, in certain proteins, as Hopkins first pointed out, free —SH groups make their appearance (12). No estimation of the number of —SH groups liberated by this reagent has yet been made. In all proteins investigated, with the possible exception of egg albumin, the appearance of —SH groups in urea solution is coincident with dissociation of the protein molecule (6, 7).<sup>1</sup> Steinhardt (30) has further shown that the functional properties of hemoglobin and pepsin are retained in urea solutions. This is a result of some significance, inasmuch as the usual concept of denaturation, implying a loss of specific properties, cannot be applied to solutions of protein in urea in spite of the apparent deep seated changes occurring in the molecule.

The study of proteins in urea solutions possesses the advantages of working in a homogeneous medium. With the porphyrindin dye, the quantitative estimation of free —SH groups at room temperature is a rapid and accurate procedure. It was thought of interest to begin this series of studies with crystalline egg albumin in urea solutions. In an attempt to find types of dispersive agents other than amides which would have the same effect as urea on the protein, the discovery was made that guanidine and

<sup>1</sup> Williams and Watson have claimed that egg albumin is dissociated in 50 per cent urea (36).

methylguanidine, or more properly the guanidonium ion and the methyl guanidonium ion, *were by far the most powerful agents of this kind.*<sup>2</sup> Either ion acted in much lower concentration than urea, and at the highest equimolecular concentrations liberated approximately 20 per cent more —SH groups. In the presence of these ions about double the number of —SH groups were found than in denaturation by either heat or precipitation. The effect of several derivatives of urea and of guanidine, such as N-methyl-urea and its isomer O-methylisourea, *as*-dimethylguanidine, and the guanidine-substituted acids was investigated and yielded comparative results of much interest.

#### EXPERIMENTAL

The egg albumin employed was crystallized six times and dialyzed until free of sulfate. It was used in several concentrations, each concentration being determined by both nitrogen and dry weight analyses. Two different preparations of protein were used.

The porphyrindin dye was synthesized according to Piloty and Schwerin (26) and Kuhn and Franke (16) and made up in fresh solution before each run. It was standardized against cysteine before and after every series of measurements. The dye solution is quite stable for 1 hour, but its titer slowly drops after this time. The standardization was conducted by dissolving 25 mg. of cysteine hydrochloride in water, adding sufficient dilute  $\text{NH}_3$  to bring the pH to 7.0, and making up to a volume of 50 cc. 1 cc. of this solution reacted *stoichiometrically* with the dye (65 mg. of porphyrindin in 100 cc. of solution), requiring 0.62 cc. of the dye. The reaction is instantaneous and the end-point quite sharp. In order to see whether this reaction is stoichiometric for more complex sulfhydryl compounds, glutathione and cysteinylcysteine (9) were titrated with the dye. Table I illustrates the quantitative nature of the reactions.

Inasmuch as the titrations of the protein were conducted in urea and in guanidine solutions, the above titrations of sulfhydryl compounds were repeated in the presence of these two substances.

\* Svedberg (32) has stated that guanidine hydrochloride has a strong effect in dissociating the molecule of *Helix* hemocyanin.



Exactly the same results were obtained, however, and the possibility of urea or guanidine having any effect on the dye may be ruled out.

The solutions of egg albumin, always at pH 7.0, at various concentrations were first tested with dye and gave a negative test for sulfhydryl. When treated with either urea or guanidine hydrochloride, the maximum number of —SH groups for each concentration of these reagents appeared within a half an hour at 25°, and further standing up to 3 hours showed neither an increase nor a diminution in this number of groups. The titrations were, therefore, performed at an interval of 45 minutes from the time of mixing the protein solution with the reagents. The pH of the solutions was always at 7.0 and the temperature 25°.

TABLE I

*Reaction of Porphyrindin with —SH Compounds*

Porphyrindin, 65 mg. in 100 cc. of solution; cysteine hydrochloride, 25 mg. in 50 cc.; glutathione, 43.6 mg. in 50 cc.; Cysteinylcysteine hydrochloride, 42.2 mg. in 50 cc. All reactions at pH 7.0.

Substance (1.0 cc.)	Dye calculated	Dye found
	cc.	cc.
Cysteine.....	0.62	0.62
Glutathione.....	0.62	0.60
Cysteinylcysteine.....	1.24	1.22

The titrations for each concentration were performed in quadruplicate and were performed again the following day. In all cases the end-points were checked by the nitroprusside reaction. The readings among the quadruplicates at each point checked among themselves to within a drop of the dye solution. Under the conditions employed, the dye is rapidly decolorized by the protein —SH groups and each reading requires no more than a minute or two. As a further partial check, the color developed by the protein solutions at several points with nitroprusside was compared with equivalent amounts of cysteine. The agreement in all cases was excellent.<sup>3</sup>

<sup>3</sup> It is hardly probable that the dye would react with other types of reducing groups in the protein. Such groups, involving tyrosine and tryptophane radicals, as Mirsky and Anson (22) point out, only begin to

The first experiments to be attempted were concerned with the effect of varying the amounts of urea in solutions of protein at two different concentrations. Table II illustrates the results obtained. It is apparent that the relative percentage of —SH groups liberated by a definite amount of urea is not affected by the protein concentration, although the relative volumes of the solutions studied were quite different. The results are always finally expressed in terms of cysteine concentration, or cysteine per hundred gm. of protein.

TABLE II  
*Effect of Various Urea Concentrations*

Protein concentration	Urea added	Dye	Cysteine
<i>per cent</i>	<i>gm.</i>	<i>cc.</i>	<i>per cent</i>
7.7 (Protein solution 1.0 cc.)	1.0	1.35	0.97
	0.9	1.20	0.85
	0.8	1.15	0.81
	0.7	0.81	0.57
	0.6	0.37	0.27
	0.5	0.05	0.04
	0.4	0	0
	2.0	1.35	0.97
3.85 (Protein solution 2.0 cc.)	1.8	1.20	0.85
	1.6	1.15	0.81
	1.4	0.85	0.61
	1.2	0.38	0.27
	1.0	0.05	0.04
	0.8	0	0

On a molar basis, 1 gm. of urea is equivalent to 1.6 gm. of guanidine hydrochloride. The effect of the latter substance on a solution of egg albumin is described in Table III. It is observed that the effect is strikingly different from that of urea. First of all, it is apparently stronger, more —SH groups are liberated, and, secondly, the effect begins at much lower concentrations of guanidine. Moreover, the maximum effect extends over a wide range of guanidine concentration.

make their presence felt at pH 10 and, moreover, react very slowly with ferricyanide and not at all with cystine or phosphotungstate. It is certain in any case that they would not exhibit a nitroprusside reaction.

The effect of liberating —SH groups just begins with urea when 0.5 gm. is added to 1 cc. of the protein solution. With guanidine hydrochloride, this effect becomes evident when the concentration is equivalent to 0.25 gm. of urea per cc. of protein solution. At the lowest effective concentrations, guanidine is thus twice as potent as urea.

It was further observed that following oxidation by the dye, the higher concentrations of protein often set to a gel. In the presence of urea, these gels were clear and translucent; those in the presence of guanidine were invariably milky and turbid.

A further series of experiments was set up to determine more closely the relation of protein concentration to the amount of sulfhydryl liberated. Table IV presents the results over a fairly wide range of protein concentration. In all, the urea concentration was invariably 1 gm. per cc. of protein solution, the guanidine hydrochloride 1.6 gm. per cc. of protein solution, and thus equimolar amounts.

The results may be consistently interpreted by the fact that, at least within the range of concentrations investigated, the proportion of —SH groups liberated by either urea or guanidine is independent of the protein concentration. Comparison of the results with urea and guanidine at equimolar concentrations shows that the effect induced by the latter is consistently greater than that by the former. The difference between the average values for each substance amounts to nearly 20 per cent.

An attempt was made to estimate the —SH groups liberated by the heat denaturation of egg albumin at pH 7.0. 2 cc. of 3.78 per cent solution of the protein were set in a boiling water bath for half an hour, then quickly cooled, and titrated with the dye. There was found 0.50 per cent of cysteine, which may be compared with the result of Todrick and Walker of 0.63 per cent (34) and the results of Mirsky and Anson (21, 22) ranging from 0.55 to 0.62 per cent. Kuhn and Desnuelle found 0.58 per cent for this protein (15).<sup>4</sup>

It was thought of interest to observe the magnitude of the effect evoked by various derivatives of urea and of guanidine.

<sup>4</sup> When the protein solution is heated in the presence of urea or guanidine, the same number of —SH groups is liberated as in the unheated protein treated with these substances.

TABLE III

*Effect of Guanidine on Egg Albumin*

Protein concentration, 3.85 per cent; protein solution, 2.0 cc. in each case.

Guanidine hydrochloride added	Dye	Cysteine
<i>gm.</i>	<i>cc.</i>	<i>per cent</i>
3.20	1.80	1.28
2.20	1.80	1.28
1.60	1.80	1.28
1.20	1.80	1.28
1.00	1.80	1.28
0.80	1.80	1.28
0.60	Gel	
0.40	0.25	0.18
0.30	0	0

TABLE IV

*Relation of Protein Concentration to -SH Produced*

Protein concentration	Protein solution	Dye		Cysteine	
		Urea	Guanidine	Urea	Guanidine
<i>per cent</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>per cent</i>	<i>per cent</i>
7.7	1.0	1.35		0.97	
5.45	2.0	2.15	2.35	1.10	1.10
4.36	2.0	1.54	1.91	0.98	1.20
3.85	2.0	1.35	1.80	0.97	1.28
3.78	2.0	1.38	1.65	0.99	1.10
2.72	2.0	1.00	1.35	1.00	1.31
1.09	2.0	0.39	0.50	1.00	1.25
Average.....				1.00	1.24

TABLE V

*Effect of Derivatives of Urea and of Guanidine*

Protein solution (5.45 per cent) 1.0 cc. in each case.

Substance	Weight of substance	Dye	Cysteine
	<i>gm.</i>	<i>cc.</i>	<i>per cent</i>
Urea.....	1.0	1.05	1.06
N-Methylurea.....	1.23	1.02	1.02
O-Methylisourea hydrochloride....	1.84	1.04	1.05
Guanidine hydrochloride.....	1.60	1.19	1.20
Methylguanidine hydrochloride....	1.80	1.18	1.19
as-Dimethylguanidine hydrochloride.....	2.00	0.75	0.75

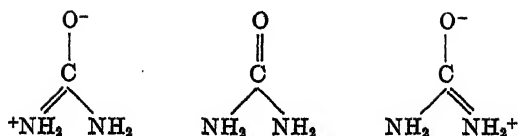
Table V lists the results obtained with equimolecular concentrations of urea, N-methylurea, and the hydrochlorides of O-methylisourea, guanidine, methylguanidine, and *as*-dimethylguanidine. The effect of urea is similar to that of its derivatives, guanidine and methylguanidine possess an apparently equal effect, whereas asymmetric dimethylguanidine is relatively weaker.

Inasmuch as the protein solution had in all cases been brought to pH 7.0 with dilute  $\text{NH}_3$ , there was necessarily always  $\text{NH}_4\text{Cl}$  present in solutions containing the hydrochlorides of the molecules. Svedberg (32) has shown that  $\text{NH}_4\text{Cl}$ , but not other chloride salts, augments the dissociation of certain proteins by weakly effective agents. In order to eliminate the possibility of this salt entering into the action of the hydrochlorides described in Table V, the experiments were repeated in protein solutions which had been brought to neutral pH with dilute  $\text{NaOH}$ . In all cases the results were identical with those in Table V. Furthermore, ammonium chloride was added to protein solutions containing the minimum amounts of urea, N-methylurea, and guanidine hydrochloride which were effective in producing sulfhydryl groups in the protein. In no case was any increase in these groups apparent.

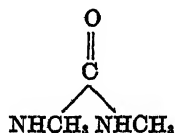
Finally, it may be mentioned that the following substances produced no sulfhydryl groups in protein solutions: arginine, N-acetylarginine, homoarginine, anhydrodiguanidocystine, glycocyamine, creatine, and creatinine.

#### DISCUSSION

Although the effect produced on the protein by solutions of urea, guanidine, and their derivatives is very striking, a simple explanation of such effect appears rather difficult at present. Any attempt to interpret the differences in the magnitude of the effects produced by the various substances must take into account the various forms in which each may occur in solution. Urea and N-methylurea exist as electrically neutral molecules; O-methylisourea, guanidine, methylguanidine, and *as*-dimethylguanidine exist as positively charged ions. All of these substances, however, possess one property in common, the capacity of existing in several resonating forms in solution. Urea, for example, may be represented as resonating among the following three structures.

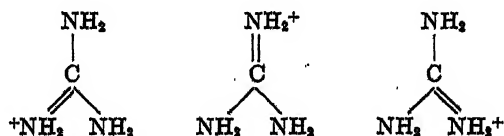


The thermochemical behavior of the amides (25), their high dipole moment values (17), and their influence on acidic dissociation (38) are best interpreted by the effect of such resonating forms. Replacement of hydrogen by methyl, as in N-methylurea, might conceivably tend to hinder the double bond from migrating to the methylated nitrogen, owing to the greater electronegativity of carbon over hydrogen. With asymmetric N,N'-dialkyl substitution this hindrance might be expected to be somewhat greater. A very much greater effect would be expected for symmetrical N,N'-dialkyl substitution, for here the resonance of the double bond would be considerably restricted, and the neutral form



would be much more important than the other two charged forms. N-Methylurea, as well as its isomer, O-methylisourea, has the same effect as urea on the protein (Table V), whereas symmetrical N,N'-diethylurea according to Hopkins (12) has no effect whatever.

Guanidine in the form of the guanidonium ion, like urea, resonates among the following three structures (25).



Substitution of a methyl group for hydrogen in this ion, as in the analogous case of urea, leads to the same effect on the protein as the parent substance produced (Table V). On the other hand, the *as*-dimethylguanidonium ion, in which resonance may be expected to be more restricted, actually shows a distinctly smaller

effect on the protein than the unsubstituted or the monosubstituted ions (Table V).<sup>5</sup>

Egg albumin possesses a total sulfur percentage of 1.62 according to Osborne (23) and 1.60 according to Baernstein (3). The former investigator also estimated the alkali-labile sulfur, presumably cystine and cysteine sulfur, to amount to 0.49 per cent; Zahnd and Clarke found 0.44 per cent (37). The percentage of cysteine based on the labile sulfur data would then be respectively 1.84 and 1.66 for this protein. No methods have hitherto been devised to determine what proportion of the labile sulfur in the native protein is cysteine or cystine. In any case, treatment with urea exposes some 60 per cent, treatment with guanidine 67 to 73 or approximately 70 per cent of the total alkali-labile sulfur content. However, part of the labile sulfur undoubtedly must belong to cystine, but the results of this paper show that only a relatively small amount of this amino acid must exist in egg albumin. By far the greater amount, up to about 70 per cent of the labile sulfur, must belong to cysteine.

From the latest value of 40,000 for the molecular weight of egg albumin (18), it would appear that there are 6 atoms of alkali-labile sulfur per molecule of this protein. On the basis of approximately 70 per cent of this type of sulfur being due to cysteine, it would appear that 4 of the 6 atoms of sulfur, or 67 per cent, were due to cysteine. This would imply that in a molecule of egg albumin, there existed at least 4 molecules of cysteine, which are revealed in solutions of guanidine. Since heat or precipitation denaturation liberates about half as much cysteine as does guanidine, it would appear that 2 molecules of cysteine are revealed by the former processes.

The manner in which the sulfhydryl groups of the native proteins are masked presents a unique problem. Astbury and Dickinson (2) have shown that the globular proteins when de-

<sup>5</sup> Edsall has shown in an extensive investigation of the Raman spectra of urea and guanidine derivatives (8) that the spectra of urea and the guanidonium ion are strikingly similar; further, the spectra of N-methylurea, the N-methylguanidonium ion, and the ion of O-methylisourea are all also quite alike. Since the magnitude of the protein effect produced by these substances is different for the urea and for the guanidine derivatives, it is clear that the vibrational bond energies, responsible for the identical spectra, play little or no rôle in the protein —SH effect.

natured exist in the  $\beta$ -keratin or stretched type of configuration. In the native state, such proteins may be assumed to exist in a coiled or contracted configuration. Several forces, notably those existing between polar groups on the side chains, can be considered as supplying the binding agents for the contracted state of the native protein. Among such polar groups, the —SH group must, at least for egg albumin, take an important place. With what type of grouping it may be linked in the native protein, in such a way that it is chemically inert, is not at present clear. The investigations of Schubert (27) on the semimercaptal linkages are suggestive in this respect. In any case, no matter in what order of events the phenomenon occurs, the action of urea or guanidine probably results in a transition of the contracted to the extended configuration of the protein, the original binding forces, including —SH groups, are dissipated, and the latter may then be chemically estimated. The effect of dissipating these binding forces is evidently very much greater through guanidine than through urea. The problem, therefore, which presents itself most insistently is to find that type of linkage involving mercaptides which is dissolved by urea or guanidine.<sup>6</sup>

#### SUMMARY

1. The use of the high potential dye, porphyrindin, for the estimation of sulphydryl groups in proteins has been extended to the study of egg albumin in solutions of urea, guanidine, and their derivatives at pH 7.0.

2. Egg albumin in solutions of 16.6 mM of urea added per cc. of protein solution showed a cysteine content amounting to 1.00 per cent. In equimolar solutions of guanidine hydrochloride, the cysteine content amounts to 1.24 per cent. These values

\* Some authors believe that the process of denaturation liberates sulphydryl groups by the hydrolysis of the S—S linkage of cystine. Speakman has interpreted the behavior of wool fibers in steam on this basis (29). Whatever may be the behavior of keratins on heating, it is doubtful whether urea and guanidine act on protein S—S linkages in such a way as to form —SH groups. No such reaction is known for simpler dithio compounds and attempts to find —SH groups on treatment of a number of cystine derivatives have proved fruitless. There is no effect whatever of urea or guanidine on substances such as cystinylcystine, anhydrocystinylcystine, cystinylglycine, or cystinylidiglycine.



are about double those reported for heated or precipitated egg albumin. Not only does guanidine possess a stronger effect in liberating sulfhydryl groups than urea, but it shows the maximum capacity at relatively lower concentrations. The percentage of sulfhydryl groups demonstrated in these solutions is independent of the protein concentration and depends only on the concentrations of urea or of guanidine.

3. The cysteine calculated from the present results amounts at the most in urea to some 60 per cent, in guanidine to about 70 per cent of the total alkali-labile sulfur of egg albumin. This would indicate that the greater amount of the alkali-labile sulfur belonged to cysteine, a much smaller amount to cystine. Of the 6 atoms of alkali-labile sulfur in the molecule of this protein, 4, or 67 per cent, apparently belong to cysteine.

4. The effects of urea, guanidine, and their derivatives are briefly discussed in terms of the resonance of these molecules. In cases like *sym*-diethylurea or *as*-dimethylguanidine, where resonance may be expected to be considerably restricted, little or no effect on the protein seems to be produced.

5. Neither urea nor guanidine has any effect on various complex peptides of cystine. It is suggested that the effect of the former substances is not on disulfide bonds, but is concerned with a dissolution of linkages within the intact protein which involve the sulfhydryl groups of cysteine. It would appear probable that such linkages were at least in part responsible for the native state of the protein.

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# STUDIES ON THE PHYSICAL CHEMISTRY OF CYSTINYL PEPTIDES

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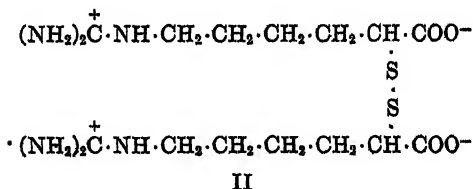
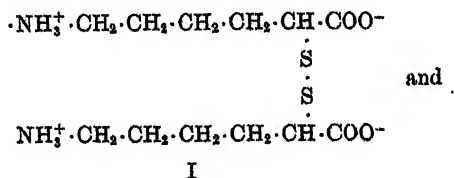
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An exact and explicit theory of the dielectric constant is lacking for polar liquids, but as the result of an analysis of a large body of data there is very strong evidence (1) that the dielectric constant is a linear function of the polarization per unit volume, a conclusion which has received added support in recent theoretical work of Onsager (2) and of Van Vleck (3). This interpretation forms the basis of an understanding of the extensive dielectric constant measurements on amino acids, peptides, and related molecules in aqueous solution and in other polar solvents, and has led to a great deal of information about their polarizations.

As a result it appears that for those amino acids and peptides in which as dipolar ions there is only one  $\text{NH}_3^+$  and one  $\text{COO}^-$  group, separated by a straight chain, the polarization is very nearly independent of everything but the length of the chain between the charges and is in fact closely proportional to it. In such highly polar molecules the optical polarization is only a very small fraction of the total. Therefore, since the dipole polarization is proportional to the mean square value of the moment, and since in dipolar ions the moment is equal to the product of the distance between the charges multiplied by the value of the elementary charge, it follows that the mean square distance between the ends of the chain, where the charges are located, is proportional to the length of the chain. Now this is exactly what is predictable on statistical grounds if there is complete free rotation about the single bonds connecting the various atoms in the chain (4, 5), and may be taken to mean that there is such

free rotation. The same results are obtained with aliphatic betaines and other similar dipolar ions, and dielectric constant studies on alicyclic amino acids, consisting of cyclohexane derivatives, have shown that in these molecules also there is a very large amount of free rotation (6).

In the case of dipolar ions containing more than one pair of charges the situation in regard to free rotation is not so clear. There are now published data for three substances of this type, each containing two  $\text{NH}_3^+$  and two  $\text{COO}^-$  groups: lysylglutamic acid,  $\epsilon, \epsilon'$ -diaminodi( $\alpha$ -thio-*n*-caproic acid) and  $\epsilon, \epsilon'$ -diguandidodi( $\alpha$ -thio-*n*-caproic acid) (7, 8). The data, however, have not



hitherto been discussed in terms of free rotation. The first of these compounds presents a somewhat complicated case, owing to the overlapping of the component dipoles, and the analysis of it will be reserved for a subsequent paper. The two others offer a simpler problem. In each the molecule consists of two parts connected by the S—S linkage, each containing a  $\text{COO}^-$  and an  $\text{NH}_3^+$  group. The structures of the 2 molecules may be written, respectively, as shown in Formulas I and II.

Either one is subject to the following analysis. The moment of the molecule is equal to the vector sum of the two equal moments belonging one to each half, and the dipole polarization is proportional to the mean square value of this sum. Now if there is free rotation about the valence bonds involving the sulfur atoms as well as about the C—C linkages, we should expect the

two halves of the molecule to be oriented very nearly at random with respect to one another. Consequently, since the mean square sum of any number of vectors arranged at random is equal to the sum of the squares of the vectors, the mean square moment of the whole molecule should be equal to twice the mean square moment found in each half. This means that the dipole polarization of the molecule should be the sum of the two equal dipole polarizations belonging one to each half. If we designate the substance by R—R and the molecule resulting from the splitting of the sulfur linkage by R we may say therefore that the polarization of a given solution of R—R should be the same as that of a solution of twice the concentration of R.<sup>1</sup>

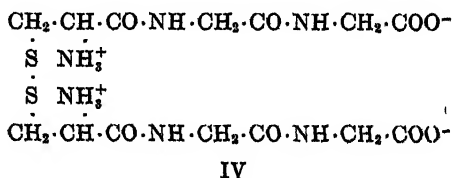
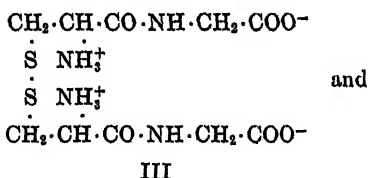
This simple conclusion provides a very easy test for free rotation in these substances. The dielectric constant, since it is linear in the polarization, should be the same for a solution of a given concentration of R—R as for a solution of twice that concentration of R, provided we neglect minor effects involving the volumes. In other words the dielectric increment, *i.e.* the increase of dielectric constant per mole of solute per liter, for R—R should be twice that for R. Each half of the  $\epsilon, \epsilon'$ -diamino acid is an  $\epsilon$ -amino acid for which the dielectric increment is close to 75. The ideal value for the dielectric increment of this molecule on the assumption of free rotation is therefore 150. Each half of the  $\epsilon, \epsilon'$ -diguanido acid contains the charged guanidine radical. In this radical, on the basis of resonance, we should expect the positive charge to behave as if located at the center of the carbon atom, a conclusion justified by dielectric constant measurements on creatine and glycocyamine, both of which molecules have dielectric increments characteristic of a  $\beta$ -amino acid (8). For this reason we may regard each half of the diguanido compound as equivalent to a  $\beta$ -amino acid, with a dielectric increment of about 87. The increment for the diguanido molecule should therefore be about 175, if there is free rotation. The observed dielectric increments of the diamino and diguanido compounds are respectively  $131 \pm 3$  and  $150 \pm 10$ . The agreement is about as close as would be

<sup>1</sup> Since the optical polarizations are themselves additive, this result does not depend on neglecting the very small contributions due to these, but it does depend on neglecting changes of dipole moment involved by splitting the S—S linkage. The effects of this must certainly be very small.

expected, in view of the fact that in the calculations we have necessarily neglected the steric interference of the two halves of the bulky molecules, and it affords strong evidence that there is a very high degree of free rotation in both these compounds. If the molecules were stabilized in the configuration of maximum moment (component dipoles parallel), the dielectric increment should be 4 times, not twice, the value for each half; if in the configuration of zero moment (component dipoles antiparallel), the increment should be negative.

### *Free Rotation in Cystinyl Peptides*

In relation to this problem of free rotation we have recently studied two peptides, one a double dipeptide, the other a double tripeptide, each containing two  $\text{NH}_3^+$  and two  $\text{COO}^-$  groups. These are cystinyldiglycine and cystinyldidiglycine, the structures of which may be written, respectively, as in Formulas III and IV. Formula III was prepared by the method of Loring



and du Vigneaud (9); the preparation of Formula IV has already been described by Greenstein (10).<sup>2</sup>

The dielectric increments ( $\delta$ ) and partial molal volumes ( $V$ )

<sup>2</sup> The titration experiments recorded in this paper revealed the presence of a minute amount of alkali bound to the cystinyldidiglycine. When this was removed by the addition of an equivalent amount of acid, the purified peptide crystallized in long prisms, had a melting point of  $181^\circ$  instead of  $145^\circ$  as given previously (10), and was only slightly soluble in water.

of both were determined in water at 25° and are given in Table I, together with values of the dielectric increments and the range of concentration (in moles per liter) covered by the dielectric constant measurements.

The dielectric constant measurements were made with a resonance method (11) at a wave-length of about 6.5 meters. They were very sharp and consistent for cystinyldiglycine. The cystinyldidiglycine solutions had considerable conductivity, owing to traces of ionic impurities which made it necessary to work at high dilutions. The calculated values of the dielectric increments are based on the assumption of free rotation and in accordance with the treatment given above are taken as twice the value of a dipeptide (70.5) for cystinyldiglycine and twice the value of a tripeptide (120) for cystinyldidiglycine. The value of

TABLE I  
*Dielectric Constant Increments and Molal Volumes of Cystinyl Peptides*

Peptide	Increment found (δ)	Increment calculated (δ)	Concentration	Molal volume
(1)	(2)	(3)	(4)	(5)
			<i>mole per l.</i>	<i>cc. per mole</i>
Cystinyldiglycine.....	139 ± 3	141	0.0129–0.0299	194 ± 0.5
Cystinyldidiglycine.....	250 ± 20	240	0.00216–0.00605	269 ± 1

120 for the dielectric increment of triglycine is taken as the mean of Devoto's value of 128 and Wyman's value of 113. The agreement between the observed and calculated values of the dielectric increments of these two peptides is extremely close and any discrepancy lies within the experimental error of the measurements. Here again, therefore, there is very strong evidence of free rotation.

The partial molal volumes of both peptides give evidence of electrostriction when compared with the volumes calculated for the uncharged molecules from the data on the volumes occupied by the various component groups. In calculating the volumes for the uncharged forms of both peptides we start with dithiodiacetic acid, which has a partial specific volume of 113.2 cc. in water at infinite dilution (7). The volume of the uncharged form of cystinyldiglycine is obtained by adding to this twice the volume



of the CO-NH unit (20 cc.), 4 times the value of the CH<sub>2</sub> group (16.3 cc.), and twice the value of the NH<sub>2</sub> group (7.7 cc.), and subtracting twice the atomic volume of hydrogen (3.1 cc.). The result is 227.6 cc. The volume of the uncharged form of cystinyldidiglycine, calculated by the same procedure, if the different numbers of groups involved are taken into account, is 300.2 cc. These two volumes, together with the observed volumes listed in Table I, Column 5, give an electrostriction of 34 cc. for the doubled dipeptide and 31 cc. for the doubled tripeptide. The former value is very close to twice Cohn's (12) value for diglycine (16.1 cc.), as would be expected; the latter value is nearly twice

TABLE II  
*Apparent Dissociation Constants of Cystinyl Peptides*

Peptide (1)	T (2)	pK' <sub>1</sub> (3)	pK' <sub>2</sub> (4)	pK' <sub>3</sub> (5)	pK' <sub>4</sub> (6)	pI (7)
	°C.					
Cystinyldi- glycine....	38	3.21 (0.02)	3.21 (0.02)	6.36 (0.03)	6.95 (0.03)	4.8
"      "	25	3.21	3.21	6.75	7.34	5.0
Cystinyldi- diglycine..	38	3.29 (0.02)	3.29 (0.02)	6.01 (0.06)	6.87 (0.02)	4.7
"      "	25	3.29	3.29	6.39	7.26	4.9
Diglycine....	25	3.14		8.07		5.60
Triglycine...	25	3.26		7.91		5.58

that for triglycine (16.1 cc.), a result also consistent with expectation.

*Apparent Dissociation Constants of Cystinyl Peptides*

We have also carried out titrations of these two peptides with a glass electrode and determined values of the four acidity constants of each. The results are given in Table II. In Columns 3 to 6 are listed the mean values of the acidity constants (pK'<sub>1</sub> ... pK'<sub>4</sub>)<sup>\*</sup> at 38°, calculated from the individual determinations

\* It should be particularly borne in mind that these pK values refer not to the dissociation constants or the equilibrium constants but to the acidity constants as defined by Brønsted. These are the constants in terms of which most of the previous results have been expressed.

by the method of Hastings and Van Slyke (13). These values are in each case followed by values of the mean deviation from the mean, regardless of sign, enclosed in parentheses. Columns 3 to 6 also contain the  $pK'$  values reduced to  $25^\circ$  by means of the van't Hoff equation with  $\Delta H = 0$  for the  $-\text{COOH}$  group and  $\Delta H = 10,000$  for the  $-\text{HN}_3^+$  group. These are given to facilitate comparison with the values for other peptides found in the literature. In Column 7 are listed the isoelectric points calculated for the two temperatures by means of the equation (14)

$$pH = \sqrt{\frac{K_1 + K_2}{K_3 + K_4}} \cdot K_3 K_4$$

In the last two rows of Table II are included for comparison the constants for diglycine and triglycine (15). Since these molecules are dipolar ions,  $pK_1$  and  $pK_2$  represent the dissociation of the carboxyl,  $pK_3$  and  $pK_4$  that of the amino groups. In Fig. 1 the titration curves of the peptides are drawn; the points are those experimentally determined, the curves are theoretical and based on the constants chosen.

Several interesting points emerge from the data of Table II. The constants for the two carboxyl groups of the cystinyl peptides are identical, a result to be expected if the groups were sufficiently separated to be independent of one another. The constants for the amino groups, although not identical, are nearly so, which also accords with approximate independence of the groups. Since the amino groups of the peptides are nearer to each other than the carboxyl groups, it would be expected that there would be some slight interaction of the former. The independence, or approximate independence, of the dissociating groups is consistent with free rotation about the S—S linkage and random mutual orientation of the two dipolar arms of each molecule.

The enormous influence of the S—S linkage on the dissociation of the adjacent amino groups may be illustrated by comparing the cystinyl peptides with diglycine and triglycine. Whereas the simple glycine peptides have a  $pK'_3 \cong 8$ , the cystinyl peptides show the extraordinarily low  $pK'_3$  values for the amino group of 6.39 and 6.75. These are, by far, the lowest  $pK$  values recorded for the amino group of this type of molecule and may be compared with the  $pK_2$  of 6.8 for the  $\alpha$ -amino group in  $\alpha, \beta$ -

diaminopropionic acid (16). Proteins, in which the  $\alpha$ -amino group of cystine is free, may therefore be expected to supplement the histidine imidazole groups in combining with acid in the range of pH 6 to 7. Steinhardt (17) has interpreted the kinetic behavior of crystalline pepsin by assigning the five basic groups of this protein dissociating at pH 6.76 to the amino groups of cystine. Such a value would indeed fall within the range of the constants

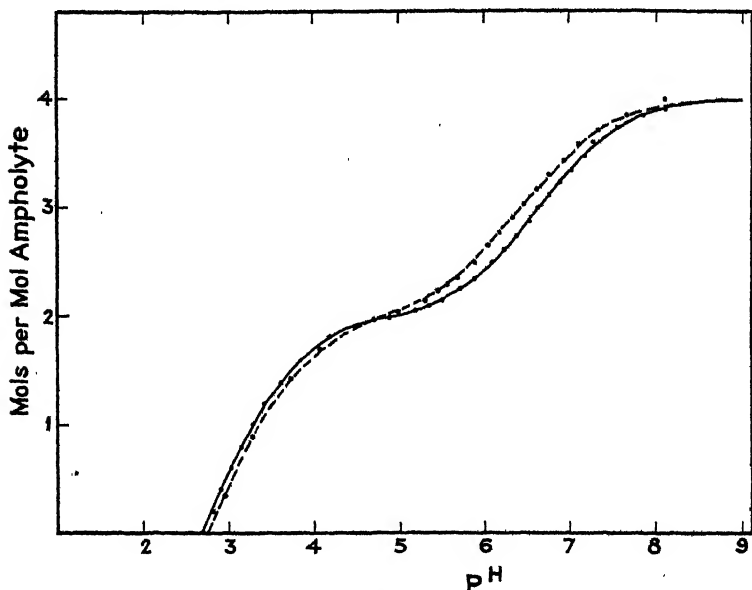


FIG. 1. Titration curves of the cystinyl peptides at 38°. The straight line refers to cystinyldiglycine; the broken line, to cystinyldidiglycine. The points are those experimentally determined; the curves are theoretical and based on the constants given in Table II.

of the amino groups of cystine peptides. On the other hand, the values for the carboxyl groups of the cystinyl peptides are not greatly different from those of the simple glycine peptides, which is undoubtedly due to the fact that in the former molecules the acid groups are far removed from the disulfide linkage. Finally, it may be pointed out that the isoelectric points of the cystinyl peptides, 5.0 and 4.9, fall within the range of several of the proteins such as egg albumin, serum albumin, etc.

*Comparative Behavior of Peptides and Proteins*

The peptides have always formed a class of molecules of particular interest because of their intermediate position between the amino acids and the proteins, and the study of the properties encountered particularly among the larger peptides is often regarded as furnishing a clue to the properties of the proteins themselves. It is of considerable interest, therefore, to consider these results on free rotation in the peptides in relation to dielectric constant studies of the proteins. It was shown by one of the present authors with zein (18), and it has since been found by other investigators with a variety of other proteins, that the dielectric constant of these molecules in solution passes through a region of anomalous dispersion at radio frequencies. The most accurate and exhaustive studies are the recent ones of Oncley (19) and Ferry and Oncley (20) on carboxyhemoglobin and the water-soluble proteins of horse serum. For carboxyhemoglobin the critical frequency is  $1.9 \times 10^6$  cycles; for all the serum proteins studied,  $0.24 \times 10^6$  cycles. It is clear from these results that these highly polar protein molecules in solution are being oriented as rigid dipoles with relaxation times of the order of  $10^{-7}$  second (carboxyhemoglobin) and  $10^{-6}$  second (serum proteins). Well below the critical frequency the dielectric constant of the protein solutions increases linearly with concentration, just as in the case of the amino acids and peptides; well above this frequency, it diminishes linearly with concentration, a phenomenon not observed in amino acids and peptides at any frequencies so far employed. The proteins, as an extreme case of the polypeptides, contain in the isoelectric condition a considerable number of pairs of positive and negative charges, each of which pairs may be thought of as a component dipole. It is evident, however, that these component dipoles cannot be oriented independently of one another in response to the field, as would be expected on the basis of free rotation in long polypeptide chains. In this respect there seems to be a significant difference between the proteins and the synthetic peptides such as we have studied in which the component dipoles are oriented independently.

Of course it might be maintained that the results on the peptides which we have just been considering do not necessarily mean that

the component dipoles actually are oriented independently of one another in response to the field. A possible interpretation would be that each molecule is oriented in the field as a rigid unit but that the statistical distribution of the molecules among all the possible configurations due to free rotation is such as to make the result the same as if there really were independent orientation of the component dipoles in the field. Actually, the deductions presented at the beginning of this paper as to what would be expected from free rotation were based on a statistical consideration of the configuration of the molecules in the absence of a field. The observed results at frequencies well below the critical frequency would be the same whether there was independent orientation of the component dipoles in the field or whether each molecule was oriented as a rigid unit, the mean square moment of all the molecules being that calculated for free rotation. Only in regard to the critical frequency should the two cases be different. It seems very unlikely, however, that this distinction should be realized physically. There is a good case in point in the hydroxy-decanoic acid polymers studied by Bridgman and Williams (21) and by Wyman (22). These polymers are not of course dipolar ions, but they are polar molecules soluble in benzene, in which it is possible to make rigorous calculations of moments. They possess a variable number of polar groups, depending on the polymer size, distributed along an aliphatic chain, in which there is free rotation, at intervals of 8 carbon atoms. At all frequencies employed the moments of these polymers are exactly the same, within the experimental error, as the moments calculated on the basis of free rotation, and even the largest polymer studied, having a molecular weight of 28,650, showed no evidence of dispersion at a frequency of  $4.8 \times 10^7$  ( $\lambda \cong 6$  meters) (22). This is almost certainly well above the critical frequency for such a large molecule if it was oriented as a rigid structure. It is clear, therefore, that in these polymers free rotation allows each polar group to be oriented independently of the others in response to the field. So far there has been no good evidence for dispersion for any of the peptides or amino acids, although reliable measurements extend up to frequencies of 5 to  $8 \times 10^7$ . This fact, however, throws very little light on the problem, since even these frequencies are not high enough for us to expect this. Even cys-

tinyldidiglycine which has a molecular weight of 504.2 is less than 0.01 the size of carboxyhemoglobin, and even if it was oriented in the field as a rigid sphere it would have a critical frequency of about  $2 \times 10^8$ .

The evidence suggests, certainly, that there is some real difference in type of structure between the synthetic peptides and the natural proteins. Possibly an understanding of this is to be found in the presence of some rigid three-dimensional grid in the natural proteins, such as is illustrated by the contracted  $\alpha$ -keratin structure proposed by Astbury (23) on the basis of x-ray studies.

On the other hand, the free rotation of the peptides in solution, involving a continuous series of configurations, presents a contrast to the behavior of long chain aliphatic molecules in films at liquid and solid surfaces, for here the molecules are apparently held rigidly in an extended configuration normal to the surface. Yet it is of interest to note that even in such films these molecules preserve a certain amount of flexibility, for there is good evidence (24) that they are capable of turning end for end even when very closely packed and it seems likely that this can only occur by a bending, snake-like process (25).

#### SUMMARY

We report the results of studies of certain physical properties of cystinyldiglycine and cystinyldidiglycine in aqueous solution. The acidity constants of the two COOH groups are the same in each peptide and close to those of the COOH group in glycine peptides. The acidity constants of the  $\text{NH}_3^+$  groups of both peptides are very much strengthened as a result of the influence of the nearby S—S linkage. The significance of this for the problem of protein titrations is pointed out. The partial molal volumes of both peptides show that there is electrostriction of the solvent, as in the case of other dipolar ions. The dielectric increments of both peptides are determined from dielectric constant measurements. An analysis of these results, as well as of published results for  $\epsilon, \epsilon'$ -diaminodi( $\alpha$ -thio-*n*-caproic acid) and  $\epsilon, \epsilon'$ -diguandiodi( $\alpha$ -thio-*n*-caproic acid), shows that in all 4 molecules, containing two  $\text{NH}_3^+$  and two  $\text{COO}^-$  groups, there is essentially free rotation about the single bonds. The free rotation in the peptides is discussed in relation to the phenomenon

of anomalous dispersion at radio frequencies observed in proteins, and in relation to the behavior of long chain molecules in surface films.

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## THE METABOLISM OF AMIDES IN GREEN PLANTS\*

### II. THE AMIDES OF THE RHUBARB LEAF

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The problem of amide metabolism in green plants is one phase of the broader problem of protein metabolism. Interest in the production of asparagine in plants has been general among plant physiologists since proteins were recognized to be the fundamental constituents of cell protoplasm (1), and the most significant work in this field, even today, is that of Ernst Schulze described in numerous papers subsequent to 1872 and summarized by him in 1906 (2). Schulze dealt mostly with the metabolism of seedlings, and arrived at the conclusion that, during the early stages of growth, the protein of the seed is digested to amino acids, these are subsequently deaminized, and the ammonia produced is recombined, with the aid of nitrogen-free nutrients, into the amides asparagine or glutamine. These substances are stored temporarily, to be drawn upon for the regeneration of protein in the growing parts. Translocation of the nitrogen occurs while still combined in simple diffusible substances.

Borodin (3), in 1878, first showed that the transformations of nitrogen in mature leaves, when these are cultured in water, follow a somewhat similar course and this aspect of the problem has furnished the theme of many investigations by Chibnall (4), Mothes (5, 6), Schwab (7), and others. Prianischnikow (8, 9), in 1922, contributed the suggestion that amide synthesis occurs in response to the presence of ammonia, being designed to main-

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.



tain the concentration of the ammonium ion below a level at which toxic effects may be produced, a suggestion that has proved very helpful in the interpretation of results secured in this laboratory with tobacco leaves subjected to culture under various conditions (10, 11). Tobacco leaves grown under field conditions appear to be very sensitive to ammonia produced endogenously, and rapid amide synthesis occurs, during culture of the detached leaves, when the concentration tends to increase above the normal level of approximately 1 per cent of the soluble nitrogen. If, in spite of this, the ammonia concentration rises, destruction of chlorophyll and other evidences of lethal damage soon become manifest, but whether these effects are the result of ammonia intoxication or have some other cause is unknown.

It has seemed desirable therefore to extend our observations to a species that can tolerate higher concentrations of ammonia than tobacco. Ruhland and Wetzel (12) have pointed out that plants which possess a highly acid sap, such as *Oxalis*, *Begonia*, and *Rheum* species, frequently contain much ammonia; the leaves of rhubarb (*Rheum hybridum*) were accordingly selected for our experiments. The specific points upon which information was desired were: Does the rhubarb leaf, a representative of the acid plant type (extract of petiole about pH 3.0, of blade about pH 4.0), possess an amide metabolism analogous to that of the tobacco leaf, a typical neutral plant (extract about pH 5.5)? Is the mechanism of amide synthesis in rhubarb correlated with the metabolism of the protein in a manner similar to that found in tobacco (10)? What, if any, evidence can be secured with regard to the chemical mechanism of amide formation?

Answers to these questions are of considerable theoretical importance. Ruhland and Wetzel hold that acid plants differ from neutral plants with respect to the metabolism of ammonia. In the acid plant, ammonia derived from the deamination of amino acids is merely neutralized by the organic acids already present or simultaneously produced by the oxidative reactions; such plants have only a minimal capacity for amide formation (13). Neutral plants, on the other hand, transform ammonia into amides, the mechanism being most conveniently described in terms of the detoxication hypothesis of Prianischnikow.

formation of glutamine and asparagine in tobacco leaves cultured in light arises from the deamination of amino acids of protein origin; the non-nitrogenous precursor which, in combination with this nitrogen, yields the specific substance glutamine, is, however, almost certainly a product, direct or indirect, of photosynthesis and is therefore probably of carbohydrate origin. No precise evidence of the chemical nature of the intermediate substances has, however, been secured. The literature contains many suggestions that organic acids of the malic or citric acid types may be involved in the transformations (5-7, 11, 14), but this has not yet been established.

The culture experiments with rhubarb leaves were conducted in a manner closely similar to earlier experiments with tobacco leaves (10). The technique and the analytical methods employed have been fully described elsewhere (11, 15, 16). The nature of the material introduced a complication, inasmuch as the rhubarb leaf possesses a long and fleshy petiole, and the behavior of the blade tissue during culture was widely different from that of the petiole. Accordingly, blade and petiole were analyzed separately at each stage of the experiment but the results are expressed in terms of gm. per kilo of fresh weight of each sample of whole leaf before culture. This assumes that it is possible to select samples of initially the same chemical composition and with the same relative proportions of blade and petiole tissue. The data indicate that the various lots of twenty leaf samples were selected with a standard error in initial weight that in no case exceeded  $\pm 3.5$  per cent and was usually less. The ratio of weight of blade tissue to that of the whole leaf was constant within 1 per cent for the leaves studied in 1936, but varied as much as  $\pm 5$  per cent in the leaves studied in 1937.

Table I shows that the protein in the blade tissue of rhubarb leaves subjected to culture in water underwent rapid digestion, and, at the end of 165 hours, approximately two-thirds had disappeared. The rate of digestion was not significantly influenced either by exposure of the leaves to light or by the presence of glucose (5 per cent) in the culture medium. The protein of the petiole, on the contrary, remained practically unchanged in amount during a similar period of culture in darkness, and increased slightly but significantly during culture in light.

became extremely rapid at the time evidences of chlorophyll degeneration and loss of turgidity first appeared (72 hours). In the experiments carried out in 1936, the leaves were cultured for 261 hours; protein digestion practically ceased, however, after the lapse of 165 hours, when about 29 per cent of the original amount still remained. The corresponding figures for the 1937 experiments were 30, 33, and 29 per cent under three different conditions of culture, and protein digestion in rhubarb is thus considerably more extensive than it is in tobacco leaves under similar

TABLE I  
*Protein Nitrogen and Soluble Nitrogen of Rhubarb Leaves during Culture*

The figures are given in gm. per kilo of original fresh weight of whole leaves.

Year.....	1936			1937			
Culture conditions*.....		DW	DW		DW	DG	LW
Time, hrs.....	0	165	261	0	165	165	165
Protein N							
Blade.....	1.46	0.381	0.419	1.09	0.329	0.356	0.312
Petiole.....	0.349	0.373	0.301	0.296	0.278	0.303	0.387
Whole leaf.....	1.81	0.754	0.720	1.39	0.607	0.659	0.699
Soluble N							
Blade.....	0.333	1.43	1.37	0.215	0.914	0.815	0.584
Petiole.....	0.365	0.604	0.586	0.458	0.628	0.546	0.897
Whole leaf.....	0.698	2.03	1.96	0.673	1.54	1.36	1.48

\* The letter D indicates culture in darkness, L in light. The letters W and G indicate respectively culture in distilled water or in 0.3 M (5.4 per cent) glucose solution. The 0 hour sample in the 1937 series furnishes a starting point for all three conditions of culture.

conditions, and is not influenced by light. It would appear that leaves contain proteins that differ in their resistance or availability to the action of the intracellular proteolytic enzymes. In rhubarb blades, less than one-third of the whole survives prolonged culture conditions in a form that can still be rendered insoluble by hot water.

The stability of the protein of the petiole is probably to be correlated with the fact that the petiole retained its turgidity throughout. The increase in petiole protein noted during culture

in light is doubtless an effect of synthesis from soluble nitrogenous substances translocated from the blade; translocation of nitrogen was evident in all cases, as is shown by the data for the soluble nitrogen of the petiole, but was particularly marked during culture in light. A similar behavior in leaves of *Tropæolum majus* has been recorded by Michael (17).

The most striking feature of the nitrogenous composition of rhubarb leaves is the high concentration of ammonia. Normal leaves may contain from 20 to 45 per cent of the soluble nitrogen of the blade in this form and from 50 to nearly 90 per cent of the soluble nitrogen of the petiole. These quantities are enormously greater than are found in such a plant as tobacco. We have never encountered tobacco leaves grown under field conditions that contained more than about 1 per cent of the soluble nitrogen as ammonia, although this can be materially increased by specific culture methods.

The behavior of the soluble nitrogen of the rhubarb leaves can be most concisely presented in terms of the change that occurred over a period of 165 hours of culture under different conditions. Because of the transport of nitrogen from blade to petiole, it is usually impossible to decide in what part of the leaf the transformations took place, and accordingly the separate data for blade and petiole tissues have been combined and are given in terms of the whole leaf.

Table II shows that from 7 to 20 per cent of the total nitrogen of the leaves was converted into ammonia in addition to the respective 14 and nearly 16 per cent of the total nitrogen present in this form in the two series from the beginning. The amide nitrogen also increased materially. A careful qualitative analysis of the blades of leaves from the same lot as those studied in 1937 showed that the only amide present, after more than 100 hours of culture in darkness, was glutamine, no asparagine whatever being detected. Although small quantities of acid-hydrolyzable ammonia were found, most of it must have belonged to some substance other than asparagine (18). The progression or trend in the values for apparent asparagine amide nitrogen during culture either in darkness or in light was hardly significant. Although a little asparagine may arise directly from the protein, the quantity, even after prolonged culture, must be very small.

TABLE II

*Changes in the Forms of Nitrogen\* in Whole Rhubarb Leaves during Culture for 165 Hours*

The figures are given in gm. per kilo of original fresh weight.

Line No.		1936, DW†	1937, DW	1937, DG†	1937, LW†
1	Ammonia N.....	0.148	0.463	0.304	0.301
2	Glutamine amide N.....	0.248	0.106	0.119	0.084
3	Protein N.....	1.056	0.783	0.731	0.691
4	$\alpha$ -Amino N liberated from protein‡..	0.744	0.595	0.556	0.525
5	Soluble amino N.....	0.703	0.425	0.365	0.321
6	Nitrate N reduced.....	0.000	0.061	0.040	0.022
7	Protein amino N, corrected for glutamic acid ((4) - 8% of (3)).....	0.660	0.532	0.498	0.470
8	Glutamine amide N, corrected for glutamine from protein ((2) - 8% of (3)).....	0.164	0.043	0.061	0.029
9	Soluble amino N, corrected for glutamine ((4) - 180% of (2)).....	0.257	0.234	0.151	0.170
10	Amino N transformed§ (7 - 9).....	0.403	0.298	0.347	0.300
11	Amino N transformed + nitrate N reduced (10 + 6).....	0.403	0.359	0.387	0.322
12	Total N of synthesized glutamine (twice (8)).....	0.328	0.086	0.122	0.058
13	Glutamine N + ammonia N (12 + 1).....	0.476	0.549	0.426	0.359
14	Difference (11 - 13).....	-0.073	-0.190	-0.039	-0.037

\* The total nitrogen of the 1936 leaves was 2.80 gm. per kilo, of the 1937 leaves 2.24 gm.; the ammonia nitrogen at the start was respectively 0.398 and 0.353 gm.; the organic solids at the start were respectively 85.8 and 60.6 gm. per kilo.

† For explanation of the letters, see Table I.

‡ The values for amino nitrogen liberated from protein for the 1936 leaves were determined by hydrolysis of the protein of the tissue before and after culture; the values for the 1937 leaves were calculated by multiplying the loss of protein nitrogen by 0.76. This factor is an average value for the proportion of amino nitrogen present after complete hydrolysis of purified leaf proteins, and was communicated to us by Professor Chibnall.

§ These figures show the extent of deamination of amino acids. They represent from 49 to 62 per cent of the  $\alpha$ -amino nitrogen liberated from the protein.

Accordingly the amide metabolism of rhubarb either in light or in darkness, unlike that of tobacco, is entirely a matter of glutamine formation.

The leaves studied in 1936 produced far more glutamine during culture in water in darkness than did those studied in 1937, and the quantity present at the end of the experiment represents a substantial part of the organic solids. Of the 40.0 gm. of organic solids per kilo of whole leaf in the blades at the start, 33.8 gm. remained at the end of 165 hours, the loss being mainly due to respiration. The blades then contained 0.207 gm. of glutamine amide nitrogen, or the equivalent of 2.15 gm. of glutamine, all save a trace being newly formed; this is 6.4 per cent of the organic solids. The only tissue we have examined that contained glutamine in a concentration approaching this order of magnitude

TABLE III

*Comparison of Amide Synthesis in Rhubarb and Tobacco Leaves during 93 and 95 Hours Respectively of Culture in Water*

The figures represent increases in amide nitrogen.

	Glutamine		Total amide	
	DW*	LW*	DW	LW
	gm.	gm.	gm.	gm.
Rhubarb blades (1937), per kilo whole leaf.	0.030	0.036	0.036	0.044
"    "    (1937), "    " blade tissue†.....	0.109	0.132	0.132	0.161
Tobacco leaves (1934), per kilo whole leaf‡.	0.038	0.071	0.238	0.116

\* For explanation of the letters, see Table I.

† Calculated from the mean blade-weight ratio of 0.273.

‡ Tobacco leaves of the variety used in these experiments do not have a petiole.

was a sample of beet roots from plants that had been heavily treated with ammonium sulfate for 18 days; the plants were small and had been severely damaged, but the roots contained 5.4 per cent of their dry weight as glutamine (19). Whether or not the performance of this particular lot of rhubarb leaves with respect to glutamine synthesis represents anything unusual will require more extensive investigation, but it is clear that the plant possesses an effective amide-synthesizing mechanism which is called into play when the ammonia content is substantially increased.

Table III shows that the 1937 rhubarb blade tissue, although less efficient in this respect than that collected in 1936, produced

more glutamine than an equal mass of tobacco leaves during culture for the same time either in light or in darkness. The rhubarb blades produced more total amide during culture in light, but the tobacco leaf excelled in darkness because of the rapid synthesis of asparagine.

The observation of an active amide-synthesizing mechanism in rhubarb, an "acid plant" according to Ruhland and Wetzel's classification, is in complete accord with results recently published by Schwab. Schwab (7) has found that *Oxalis deppei* leaves form glutamine and *Pelargonium peltatum* leaves form asparagine during culture under conditions similar to those we have employed, and it may therefore be concluded that the so called acid plants do not differ qualitatively in this respect from neutral plants.

Attempts to correlate the quantities of glutamine formed with the ammonia presumably liberated by oxidative deamination of amino acids derived from the protein have been moderately successful. The analytical difficulties are serious, and several assumptions must be made, since practically nothing is known about the constitution of rhubarb leaf proteins. If, however, these proteins are analogous in composition to the grass leaf proteins analyzed by Miller (20) in Chibnall's laboratory, they may be expected to yield about 8 per cent of their nitrogen as the nitrogen of glutamic acid, although the glutamic acid is probably combined in the native protein mainly as the amide. From analogy with the results with tobacco leaves, it seems probable that protein is digested by the intracellular proteolytic enzymes completely to the amino acid stage. Whether glutamic acid is liberated as such under these conditions, or as the amide glutamine is unknown, but the presence of glutamine-synthesizing amides in the leaves suggests the likelihood that glutamine is produced. If this be assumed, it is possible to show from the data in Table II that more than half of the amino nitrogen, exclusive of glutamic acid nitrogen derived from the protein, must have been destroyed as such (Line 10), since the soluble amino nitrogen, corrected for glutamine (Line 9), is much less than would be expected from the behavior of the protein (Line 4). If the amino nitrogen that was destroyed is transformed into ammonia and 2 moles of this ammonia combine with a non-nitrogenous precursor to yield

glutamine, the quantities of nitrogen involved can be accounted for in the 1936 leaves within 0.073 gm. The steps in the calculation, founded on the assumptions above, are shown in Lines 7 to 13 of Table II. So small a discrepancy is readily accounted for if a little nitrogen in forms other than  $\alpha$ -amino nitrogen is converted into ammonia. Closer agreement could perhaps scarcely be expected, when the analytical and sampling errors are considered.

Similar calculations made upon the leaves studied in 1937 led, in two out of three experiments, to even closer agreement, the exceptional case being entirely due to an unusually high value for ammonia in the petioles of one sample. For these leaves, it was necessary to employ the factor 0.76 to estimate the amino nitrogen liberated by enzyme hydrolysis of the protein. This factor is an average obtained by Professor Chibnall<sup>1</sup> from studies of a wide variety of purified leaf proteins.

The 1937 leaves were considerably less effective in disposing of ammonia as an amide than the 1936 leaves. In fact, it is possible to show, from data obtained after 93 hours of culture (not given in Table II),<sup>2</sup> that the whole of the glutamine present at that time may have arisen directly from the protein. The amide-synthesizing mechanism may therefore have come into definite operation only after the lapse of 93 hours. Clearly, different samples of rhubarb leaves may differ quite widely in their capacity to synthesize glutamine. Attempts to employ water-cultured rhubarb leaf blades as material for the preparation of glutamine in quantity have also been disappointing, probably owing to this variability.

The nature of the precursor that, in combination with ammonia, yields glutamine in leaf tissue is at present a matter of debate. The experiments of Suzuki (21) and of Prianischnikow (8) point to the carbohydrate of the leaf, those of Mothes (6) to the organic acids as being concerned. Studies of tobacco leaves in this laboratory (10, 11) indicated that the presence of the products of photosynthesis is essential, although no information was secured with respect to the intermediates involved. Careful

<sup>1</sup> Personal communication.

<sup>2</sup> The complete data, together with a fuller discussion than can be given here, will be presented in a forthcoming bulletin from this Station.



examination of the carbohydrates and organic acids of the present samples of rhubarb leaves was made, but no direct evidence was found to link either group of substances with the glutamine synthesis, nor was there any effect from the products of photosynthesis produced during culture in light. The failure of the leaves cultured in 5 per cent glucose in darkness to show any noteworthy increase in glutamine over the control in water, notwithstanding the facts that glucose was absorbed, ammonia was present in large quantities, and glutamine synthesis was actually in progress, suggests that glucose, at all events under the conditions of this experiment, is not directly involved in the reaction.

#### SUMMARY

The leaves of the rhubarb plant are characterized by a far more acid reaction than those of the tobacco plant, and may normally contain very considerable concentrations of ammonium ion. They possess an amide-synthesizing mechanism which responds when the ammonium ion concentration rises as a result of protein decomposition during culture of detached leaves. Glutamine is synthesized both in light and in darkness; different specimens may, however, vary somewhat widely in their capacity to produce this amide. One sample of blade tissue investigated became so enriched as to contain nearly 7 per cent of its organic solids as glutamine; other samples behaved in a manner not greatly different from tobacco leaves. Although there is no doubt of the capacity of the rhubarb leaf to produce glutamine from ammonia and some unknown carbon compound, it is quite possible that a substantial part of the newly formed glutamine may be directly derived from the protein.

It is difficult to account for glutamine synthesis in rhubarb leaves in terms of the detoxication hypothesis of Prianischnikow, since there is no apparent reason why a leaf that may contain 15 per cent or more of its total nitrogen as ammonium ion should suffer damage when this is slightly increased. Illuminating as his views may be when applied to tobacco leaves subjected to the highly artificial conditions of water culture, they do not shed much light on the behavior of rhubarb leaves in like circumstances. It is possible that amide synthesis is interposed, so to speak, as a buffer to prevent rapid fluctuations in ammonia content; to

this extent the detoxication idea may express a reality, but the fact remains that rhubarb and possibly other plants of similar high acidity are far more tolerant of ammonia than the more nearly neutral tobacco plant.

The general mechanism for ammonia production in plant tissues from deamination of amino acids of protein origin, originally suggested by Schulze, applies in the rhubarb plant, and an approximately quantitative relationship in the nature of a balance sheet between the  $\alpha$ -amino nitrogen and the products of its transformations can be drawn up. An excess of ammonia is, however, invariably found which indicates that minor quantities of nitrogen in forms other than  $\alpha$ -amino nitrogen may become involved in the oxidation reactions.

No positive evidence of the nature of the precursor of glutamine has been found. Synthesis occurred in parallel experiments almost equally well in light and in darkness and is, accordingly, not dependent on the presence of the immediate products of photosynthesis as it is in tobacco. Culture in darkness on glucose solution gave rise to no marked increase in glutamine production, although evidence was secured that the glucose entered the tissues and that ample ammonia was present. Accordingly, glucose itself is probably not the direct nor the indirect precursor of glutamine in rhubarb.

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## THE ENZYMIC HYDROLYSIS OF BENZYL STEARATE AND BENZYL BUTYRATE\*

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(Received for publication, June 25, 1938)

A study of the action of pancreas lipase and liver esterase on a variety of substrates led to the observation that esters of benzyl alcohol were split with astonishing rapidity in comparison with the fats and esters ordinarily employed in such experiments. This led us to a more detailed study of the behavior of benzyl stearate and its homologue benzyl butyrate.

Benzyl stearate (m.p. 45°) and benzyl butyrate (b.p. 108–110° at 9 mm.) were treated with crude glycerol extracts of pancreas and liver in the presence of bile and with the gradual addition of ammonia as described in an earlier paper (1). Unless otherwise stated the experiments were made on 0.214 gm. of the butyrate or 0.449 gm. of the stearate emulsified in 30 cc. of a digestion mixture to which suitable amounts of enzyme were added. Hydrolysis was determined by the titration of 5 cc. portions of the system dissolved in a mixture of alcohol and ether.

*Action of Pancreas Extract*—The course of the hydrolysis with time at 40° was measured at several enzyme concentrations until almost complete disappearance of the substrate occurred. It is practically a straight line for both benzyl stearate and benzyl butyrate. Such zero order reactions have been observed with liver esterase (2) but never as far as we are aware with pancreas lipase.

The evident conclusion is that the rate of hydrolysis at this temperature is independent of the substrate concentration over a wide range. This was confirmed by a direct experiment shown in Table I.

\* Food Research Division Contribution No. 388.

The speed of hydrolysis of benzyl butyrate and benzyl stearate at temperatures lower than 40° coincides with our previous experience that only the esters of lower or unsaturated fatty acids are hydrolyzed in the cold. However, while tristearin undergoes

TABLE I

*Alkali Required after Partial Hydrolysis of Different Quantities of Substrate with Same Quantity of Enzyme*

Time	Amount of 0.1 N KOH required after addition of			
	Benzyl stearate		Benzyl butyrate	
	0.2 mm per titration	0.4 mm per titration	0.2 mm per titration	0.4 mm per titration
	cc.	cc.	cc.	cc.
10	0.49	0.61	0.53	0.55
20	0.90	1.05	1.00	1.00
30	1.37	1.48	1.37	1.43
40	1.80	1.95	1.72	1.90
Enzyme used.	0.16		0.08	

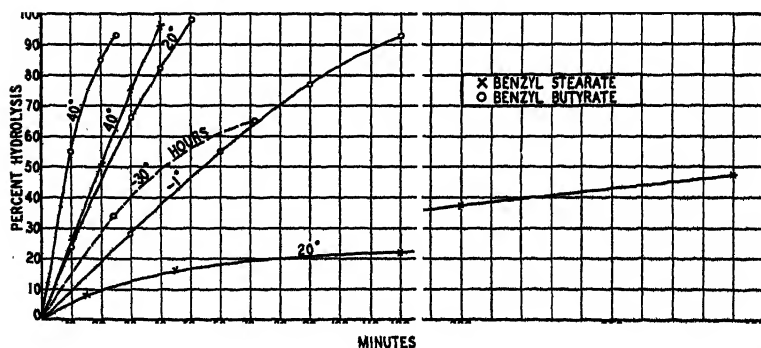


FIG. 1. Hydrolysis of benzyl stearate and benzyl butyrate (1.2 mm) by 1.0 cc. of pancreas extract at various temperatures.

almost no splitting at 20°, benzyl stearate is hydrolyzed at this temperature with fair rapidity.

The butyrate, on the other hand, continues to hydrolyze rapidly when the system is frozen solid. It is apparent that the inertness of stearic esters at low temperatures is only comparative and varies with the alcohol.

A comparison of the curves shown in Fig. 1 for benzyl stearate and benzyl butyrate at several temperatures shows that the form of the curve, that is the apparent kinetics of the reaction, changes with the temperature. As the latter decreases, the picture of a zero order reaction disappears and the curves become more and more bent until they resemble the curves for lipase action observed

TABLE II

*Speed of Hydrolysis Observed with Various Quantities of Enzyme*

The enzyme units represent cc. of 0.1 N acid formed per minute, per cc. of enzyme preparation.

Benzyl butyrate			Benzyl stearate		
Enzyme	Time	Enzyme unit	Enzyme	Time	Enzyme unit
cc.	min.	cc.	cc.	min.	cc.
1.00	10	0.09	1.00	10	0.05
	20	0.08		20	0.05
	25	0.07		30	0.05
0.50	10	0.11	0.50	40	0.05
	20	0.10		10	0.06
	30	0.09		20	0.06
	40	0.09		30	0.06
0.25	15	0.10	0.25	50	0.06
	30	0.09		65	0.06
	55	0.09		15	0.06
	80	0.09		35	0.06
0.125	30	0.09	0.125	65	0.06
	70	0.09		115	0.06
	130	0.08		60	0.05
	180	0.09		120	0.05
0.0625	60	0.11		210	0.05
	120	0.10		300	0.05
	215	0.09			
	335	0.09			

with the higher fats. The only apparent difference between the butyrate and the stearate in this regard is the temperature level at which the change of form takes place. The effect of temperature on the hydrolysis of higher and lower esters thus appears to be referable to no fundamental difference between the two types of substrates, but it leads to a very great practical difference in the results obtained under ordinary laboratory conditions.

It is well recognized that any consideration of the kinetics of lipase action is greatly complicated by the heterogeneous nature of the system. Even if the variations with temperature observed here are considered as introducing no new factor into the problem, they furnish a good illustration of the difficulty and perhaps the futility of kinetic studies on lipase.

*Method for Estimating Pancreas Lipase*—Without regard for the kinetics involved, it is evident, however, that the straight line relationships observed at 40° offer promise of a rapid and convenient method for estimating pancreas lipase.

The speed of hydrolysis with different quantities of enzyme in the case of benzyl stearate is approximately proportional to the

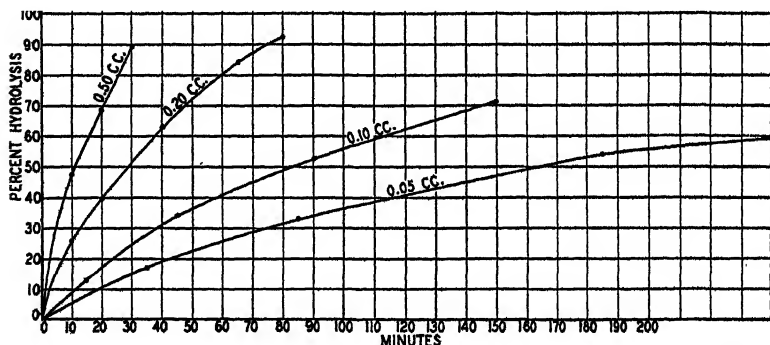


FIG. 2. Hydrolysis of benzyl butyrate at 40° by various amounts of liver extract.

amount of enzyme added. With benzyl butyrate it is very closely proportional. The latter substance is therefore suitable for a test substrate. The data presented in Table II are expressed in cc. of 0.1 N acid formed per minute, per cc. of enzyme preparation. This is a convenient form of enzyme unit because the amount of substrate may vary considerably without affecting the result, which is obviously not the case if the per cent of hydrolysis is used in the computation. Results on benzyl stearate are also given in Table II for comparison only. The variations observed with this substrate are somewhat too great to permit its use. They are probably due to mistakes of manipulation, however. It is difficult to adjust the pH of the system fast enough without

occasionally overneutralizing it. Furthermore, it is unlikely that emulsions of benzyl stearate can be reproduced as accurately with respect to particle size as those of benzyl butyrate.

*Comparison of Pancreas with Liver Extract*—In experiments at 40°, while pancreas extract hydrolyzed both the stearate and butyrate, liver extract hydrolyzed butyrate only. The course of hydrolysis found with the liver preparation is shown in Fig. 2. It is similar to that ordinarily observed with lipase, resembling but not quite fitting a monomolecular reaction. Benzyl butyrate and benzyl stearate at 40° therefore offer a method of distinguishing the action of liver from that of pancreas in hydrolysis.

#### SUMMARY

Benzyl butyrate and benzyl stearate are hydrolyzed with remarkable speed by pancreas extract, and benzyl butyrate by liver extract. At 40° the reaction velocity of butyrate hydrolysis follows distinctly different courses with pancreas and with liver preparations.

The splitting of both benzyl esters by pancreas extract at 40° is a reaction of the so called zero order, but at lower temperatures the curves are fairly typical of ordinary lipase action. Hydrolysis of the stearate is more affected by a lowering of temperature than is that of the butyrate. In common with other substrates previously reported the butyrate is split at low temperatures, and the stearate only when the temperature is relatively high. A convenient method for the estimation of lipase with benzyl butyrate at 40° is proposed.

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## CARBOHYDRATE OXIDATION IN NORMAL AND DIABETIC CEREBRAL TISSUES\*

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Investigations by various workers on the metabolism of cerebral tissue, both *in vivo* (Himwich and Nahum (1932); Lennox (1931)) and *in vitro* (Dickens and Simer (1931); Loebel (1925); Ashford and Holmes (1931)) indicate that the chief substrate for brain oxidation is carbohydrate. Moreover, unlike most tissues of the diabetic animal, cerebral tissue is able to oxidize carbohydrate, with a respiratory quotient of approximately unity, and must therefore possess some mechanism for carbohydrate oxidation which does not include insulin. Since the brain is uniquely dependent on carbohydrate for its energy supplies, the method of oxidation of carbohydrate by cerebral tissue is of particular importance.

Recent experiments have cast doubt on the validity of the theory that the formation of lactic acid is an essential stage in the oxidation of glucose. Thus, Himwich and Fazekas (1935, 1936) showed that nicotine inhibited lactate oxidation by minced cerebral tissue to a much greater extent than glucose oxidation, and concluded that glucose could be oxidized without going through a lactic acid stage. Jowett and Quastel (1937), using hydroxymalonate, reached similar conclusions. Stannard (1937), Saslow (1937), and Shorr, Barker, and Malan (1938) observed high respiratory quotients for muscle, brain, and other tissues, indicating carbohydrate oxidation, in the presence of a concentration of iodoacetic acid sufficient to abolish anaerobic glycolysis. Similarly, Baker (1938) reported practically normal brain respiration

\* Aided by a grant from Child Neurology Research (Friedsam Foundation). A preliminary communication of this work has appeared (*Am. J. Physiol.*, **123**, 6 (1938)).

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in the presence of a concentration of glyceraldehyde which completely repressed anaerobic glycolysis.

In the present communication, experiments are reported in which carbohydrate utilization was determined both by manometric and by direct chemical analysis, with normal and diabetic cerebral tissue, in the presence of nicotine.

### *Methods*

The metabolism of isolated tissue slices was studied in the Dixon-Keilin manometers, with the methods of Elliott and Schroeder (1934) and the bicarbonate-containing medium of Krebs and Henseleit (1932). All substrates were made up to the desired final concentration (0.02 M). For an experiment with rat brain, sliced cerebral cortex of four animals was usually used, carefully sampled, in each of four manometers, so as to insure uniform distribution. Tissue was excised also from either normal or depancreatized cats at least 72 hours postoperative. All results are calculated in terms of dry weight of tissue. When chemical estimations were to be made, the contents of the vessels were carefully washed out and diluted to 25 cc., and aliquots of this filtrate used. Lactic acid was estimated by the method of Friedemann, Cotonio, and Shaffer (1927), and glucose by that of Hagedorn and Jensen (1923).

### *Results*

*Glucose and Lactate Oxidation in Brain*—Table I presents the results obtained with nicotine on the metabolism of rat brain slices in the presence of either glucose or lactate. The addition of nicotine causes an inhibition of respiration, progressively greater with higher concentrations (Experiments 1 and 2). In all cases, however, at equivalent concentrations of nicotine, the inhibition of lactate oxidation is approximately twice as great as that of glucose. 0.03 M nicotine inhibits  $Q_{O_2}$  in the presence of glucose 49 per cent (average) and lactate oxidation 91 per cent (average) (Experiments 1, 2, 6, 7, 8).

There is, in addition, a striking increase in aerobic glycolysis ( $Q_A^{O_2}$ ) in the presence of nicotine, reaching values as high as +13.6. Lactic acid estimations indicated that the increased acid production could be accounted for almost entirely by lactic acid.

In Table I are also presented figures for glucose disappearance

TABLE I

*Rat Cerebral Cortex Plus Nicotine*

Experimental period 90 minutes; temperature 38°.

Experiment No.	Concentration of nicotine	Glucose, 0.02 M				Lactate, 0.02 M				Glucose disappearing for respiration, per 100 mg. dry weight of tissue			
		-Q <sub>O<sub>2</sub></sub>	R.Q.	Q <sub>A</sub>	Inhibition of Q <sub>O<sub>2</sub></sub>	-Q <sub>O<sub>2</sub></sub>	R.Q.	Q <sub>A</sub>	Inhibition of Q <sub>O<sub>2</sub></sub>	Without nicotine		With nicotine	
										Manometric	Estimated	Manometric	Estimated
	<i>mole per l.</i>				<i>per cent</i>				<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	0	15.0	0.92	+2.6									
	0.005	13.7	0.96	+3.8	8								
	0.015	10.6	0.94	+6.3	28								
	0.030	5.9	0.86	+13.0	60								
2	0					13.8	0.89	-2.5					
	0.005					10.6	0.82	+0.4	23				
	0.015					5.2	0.66		63				
	0.030					0.0			100				
3	0					13.4	0.92	-1.8					
	0.015					8.3	1.03	-1.4	38				
4	0					12.3	0.93						
	0.015					8.1	0.92		34				
5	0	14.2	0.96	-0.7		13.6	1.04	-3.2					
	0.015	10.8	0.92	+5.9	24	7.1	0.88	-0.1	48				
6*	0	15.5	0.96	0.0		15.5				2.1	1.4		
	0.03	7.7	0.98	+8.1	50	2.4			85			1.0	0.9
7*	0	15.0	0.90	+0.6		15.0				2.0	1.7		
	0.03	8.3	0.98	+8.1	45	1.8			88			1.1	1.4
8*	0	15.7	0.93	+0.7		15.7				2.1	1.7		
	0.03	8.8	0.95	+9.6	45	1.4			91			1.2	1.1

-Q<sub>O<sub>2</sub></sub> = c.mm. of oxygen consumed per mg. of dry weight of tissue per hour.

Q<sub>A</sub> = c.mm. of CO<sub>2</sub> evolved, equivalent to lactic acid, per mg. of dry weight of tissue per hour (aerobic).

\* 2½ hour experimental period.

for three experiments (Nos. 6, 7, 8). The figures listed under "Manometric" represent the amount of glucose disappearing per 100 mg. of dry weight of tissue per hour, calculated from manometric data, on the assumption that all respiration is at the ex-

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pense of glucose (1 mg. of glucose  $\approx$  747 c.mm. of  $O_2$ ). The estimated values were obtained by determining the difference in glucose concentration between the left and the right manometric vessels, corrections being applied for the amount of glucose which was converted to lactic acid. As can be seen from these data, there is fairly good agreement between the manometric and the chemically estimated results. Thus, average values for glucose utilization in three experiments are, in the absence of nicotine, 2.1 mg. (manometric) and 1.6 mg. (estimated), and, in the presence of nicotine, 1.1 mg. (manometric) and 1.1 mg. (estimated).

The respiratory quotient of brain tissue with glucose as substrate was little affected by nicotine in concentrations as high as 0.03 M, despite the fact that the respiration was inhibited as much as 60 per cent (Experiments 1, 5 to 8). In the absence of nicotine, values varied between 0.90 and 0.96, and in the presence of 0.03 M nicotine from 0.86 to 1.00. Apparently, therefore, the glucose was oxidized to completion.

Similar results were obtained on the brains of normal and diabetic cats (Table II). On the average, 0.03 M nicotine inhibited glucose oxidation 28 per cent and lactate oxidation 56 per cent in the normal brain. In the diabetic brain, 0.03 M nicotine inhibited glucose oxidation 28 per cent and lactate oxidation 58 per cent; with 0.05 M nicotine the inhibition was 35 per cent for glucose and 69 per cent for lactate. For cat brain a higher concentration of nicotine was required to produce a degree of inhibition comparable with that obtained on rat brain.

In the absence of nicotine the aerobic glycolysis  $Q_A$  of cerebral cortex from both normal and diabetic cats was found to be higher than that obtained with the rat, ranging from +0.7 to +3.9; nicotine increased these values (+3.8 to +9.9), although the increase was not so striking as that seen with rat brain. Nicotine had no significant effect on the respiratory quotients of either normal or diabetic cat brain in the presence of glucose (without nicotine, 0.93 to 1.01; with nicotine, 0.84 to 1.03).

Chemical and manometric estimations of glucose utilization by diabetic cerebral cortex are also shown in Table II. With 0.03 M nicotine, the following results (average for three experiments) were obtained: without nicotine, 1.1 mg. (manometric) and 1.0 mg. (estimated), with nicotine, 0.8 mg. (manometric) and 0.9 mg. (estimated); with 0.05 M nicotine (average of two experi-

TABLE II  
*Cat Cerebral Cortex Plus Nicotine*

Experiment No.	Con- centra- tion of nicotine	Glucose, 0.02 M				Lactate, 0.02 M				Glucose disappearing for respiration, per 100 mg. dry weight of tissue			
		-QO <sub>2</sub>	R.Q.	Q <sub>A</sub>	Inhi- bition of QO <sub>2</sub>	-QO <sub>2</sub>	R.Q.	Q <sub>A</sub>	Inhi- bition of QO <sub>2</sub>	Without nicotine		With nicotine	
										Manometric	Estimated	Manometric	Estimated
Normal													
1	0	9.1	0.95	+2.9		7.8	1.01	-0.8					
	0.03	6.7	1.00	+6.3	27	2.8	0.89	-0.5	64				
2	0	8.7	0.94	+3.8		10.7	0.88	-0.6					
	0.03	6.3	1.00	+4.9	28	4.1	1.03	-1.5	62				
3	0	7.1	0.99	+3.2		8.7	0.97	-2.0					
	0.03	5.2	1.03	+5.3	28	4.9	0.94	-0.5	43				
Average.....					28				56				
Diabetic													
1	0	8.8	0.97	+0.7		9.7	0.86	-1.0					
	0.03	5.5	0.90	+3.8	38	5.4	0.93	-0.8	45				
2	0	8.9	1.01	+1.1		9.1	0.90	-1.2					
	0.03	6.5	0.99	+5.3	27	6.3	0.93	-0.9	31				
3	0	10.2	0.98	+1.9		9.6	1.03	-2.3					
	0.03	7.0	0.84	+6.1	32	4.8	1.03	-0.3	51				
4	0	9.0	0.93	+3.9		9.4	0.98	-0.8		1.1	1.1		
	0.03	6.5	0.93	+7.1	28	1.3	0.93	-0.4	86			0.8	0.8
5	0	9.5	0.96	+3.1		10.1	0.93	-1.7		1.2	1.0		
	0.03	7.2	1.00	+5.5	24	2.1		-1.0	79			1.0	1.0
6	0	8.3	0.96	+1.8		8.6	0.90	-1.1		1.1	0.8		
	0.03	6.6	0.87	+4.9	20	3.6	1.00	+0.1	59			0.8	1.0
Average.....					28				58				
7	0	9.0	0.94	+1.1		9.0	0.94			1.2	1.0		
	0.05	6.1	0.94	+4.3	33	2.8	0.79		68			0.8	0.6
8	0	8.6	0.97	+2.2		8.6	0.97			1.2	1.0		
	0.05	5.3	0.95	+6.2	38	2.5	0.62		70			0.7	1.0
Average.....					35				69				

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ments), without nicotine, 1.2 mg. (manometric) and 1.0 mg. (estimated); with nicotine, 0.8 mg. (manometric) and 1.0 mg. (estimated).

TABLE III  
*Effect of Nicotinic Acid and Nicotinic Acid Amide on  
Rat Cerebral Cortex*

Experiment No.	Concentration	Glucose, 0.02 M				Lactate, 0.02 M			
		$-Q_{O_2}$	R.Q.	$Q_A$	Inhibition of $Q_{O_2}$	$-Q_{O_2}$	R.Q.	$Q_A$	Inhibition of $Q_{O_2}$
Nicotinic acid									
	<i>mole per l.</i>				<i>per cent</i>				<i>per cent</i>
1	0	13.5	0.93	+0.9		13.5	0.93		
	0.03	12.5	0.88	+5.4	7	4.2			69
2	0	13.9	0.96	-0.2		13.9	0.96	-0.3	
	0.03	12.8	0.96	+4.9	8	3.5	0.89	-1.5	75
3	0	14.9	0.89	+1.9		14.9	0.89		
	0.03	13.2	0.95	+5.7	11	8.4	0.77	-0.8	44
4	0					11.8	0.93	-0.6	
	0.03					7.5	0.98	-1.9	36
5	0					12.6	0.97		
	0.03					4.7			63
Average.....					9				57
Nicotinic acid amide									
1	0	13.3	1.01	-0.4		13.3	1.00	-0.4	
	0.03	12.8	0.96	0.0	4	8.4	0.72	+1.0	37
2	0	11.8	0.93	-0.6		11.8	0.93	-0.6	
	0.03	10.3	0.82	+2.8	13	10.1	0.90	-1.2	14
3	0	12.6	0.97	-2.5		12.6	0.97	-2.5	
	0.03	10.7	0.86	+3.4	15	13.5	0.93	-2.5	-7
4	0	14.8	0.93	+1.6					
	0.03	14.6	0.86	+2.0	1				
Average.....					8				15

*Effects of Nicotinic Acid<sup>1</sup> and Nicotinic Acid Amide<sup>2</sup>*—As is indicated in Table III, the results obtained with nicotinic acid at a

<sup>1</sup> Eastman Kodak Company; recrystallized from alcohol.

<sup>2</sup> Sample prepared from liver, kindly donated by Dr. M. O. Schultze of the University of Pittsburgh.

TABLE IV  
*Rat Cerebral Cortex Plus Nicotine*

Experiment No.	Concentration of nicotine	Pyruvate, 0.02 M				Lactate, 0.02 M			
		$-Q_{O_2}$	R.Q.	$Q_A$	Inhibition of $Q_{O_2}$	$-Q_{O_2}$	R.Q.	$Q_A$	Inhibition of $Q_{O_2}$
	<i>mole per L.</i>				<i>per cent</i>				<i>per cent</i>
1	0	12.3	1.34	-4.8					
	0.015	5.9	1.40	-2.9	52				
	0.030	4.7	1.20	-0.5	61				
2	0	12.5	1.32	-4.2					
	0.015	7.1		-4.3	43	5.0	1.03	-0.1	60
	0.030	2.8		-1.7	78				
3	0	12.7	1.38	-4.9					
	0.015	7.2	1.44	-2.9	43	7.4	0.90	-0.3	42
	0.030	3.3		-2.7	74				
4	0	11.2	1.34	-4.6					
	0.015	6.4	1.44	-3.6	43	5.5	0.95	-2.3	51
	0.030	1.0			92				

TABLE V  
*Rat Cerebral Cortex Plus Nicotine*

Experiment No.	Concentration of nicotine	Fructose, 0.02 M				Concentration of nicotine	Succinate, 0.02 M			
		$-Q_{O_2}$	R.Q.	$Q_A$	Inhibition of $Q_{O_2}$		$-Q_{O_2}$	R.Q.	$Q_A$	Inhibition of $Q_{O_2}$
	<i>mole per L.</i>				<i>per cent</i>	<i>mole per L.</i>				<i>per cent</i>
1	0	12.3	0.93	+1.5		0	12.8	0.33	-1.4	
	0.015	7.3	0.93	+0.9	41	0.005	12.4	0.23	-2.1	3
2	0	13.1	0.93	+0.6		0	14.8			
	0.015	4.9	0.88	+1.3	63	0.005	13.5	0.32		9
						0.015	13.1	0.47		11
3	0	13.6	0.93	+0.4		0.030	12.6			17
						0	11.3	0.37	-0.8	
						0.015	13.0	0.37	-0.1	-15
	0.030	2.0	1.00	-0.1	85					
4	0					0	13.5			
						0.03	13.1	0.33	-1.4	3
5	0					0	13.9		-0.2	
						0.03	10.1	0.31	+0.5	20



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concentration of 0.03 M are even more striking than those with nicotine, glucose oxidation being inhibited only 9 per cent and that of lactate 57 per cent. Since the toxicity of this substance is considerably less than that of nicotine, much higher concentrations are required to produce the essentially complete inhibition of lactate oxidation which is obtained with 0.03 M nicotine. Like

TABLE VI  
*Rat Kidney Plus Nicotine*

Experiment No.	Concentration of nicotine	Glucose, 0.02 M				Lactate, 0.02 M			
		$-Q_{O_2}$	R.Q.	$Q_A$	Inhibition of $Q_{O_2}$	$-Q_{O_2}$	R.Q.	$Q_A$	Inhibition of $Q_{O_2}$
	mole per l.				per cent				per cent
1	0	24.1	0.87	-1.8		31.7	0.89	-5.1	
	0.015	18.1	0.94	-1.7	25	23.6	0.91	-4.4	26
2	0	23.4	0.89	-1.7		30.4	0.90	-4.4	
	0.015	16.8	0.94	-2.1	28	25.7	0.87	-3.7	15
3	0	24.7	0.85	-0.6					
	0.03	13.6	0.87	-1.4	45				
4	0	21.7	0.89	-1.6		29.7	0.94	-5.4	
	0.03	14.7	0.92	-0.1	32	23.2	0.87	-3.7	22
5	0	18.7	0.81	-0.4		27.6	0.89	-4.6	
	0.03	11.3	0.98	-1.9	39	17.1	0.88	-2.0	38
No substrate added									
6	0	19.6	0.72	+0.9					
	0.03	11.0	0.76	-0.2	44				
7	0	20.3	0.88	-3.1					
	0.03	10.9	0.73	-0.2	47				
8	0	18.5	0.80	-0.6					
	0.06	1.8	0.72	+0.6	90				
9	0	22.3	0.81	-1.7					
	0.06	3.3	0.75	+0.7	85				

nicotine, however, nicotinic acid stimulates glycolysis. Nicotinic acid amide was found to have little effect at this concentration, the inhibition of glucose oxidation being on the average only 8 per cent, and that of lactate only 15 per cent. Glycolysis was not affected.

*Various Substrates*—The effect of nicotine on the oxidation by

rat cerebral cortex of pyruvate, succinate, and fructose has also been studied. Pyruvate oxidation (Table IV) was found to be markedly inhibited, 45 per cent (average) for 0.015 M nicotine and 76 per cent (average) for 0.03 M nicotine. In several experiments, also listed in Table IV, the effect of nicotine on lactate and pyruvate oxidation was directly compared; a striking degree of correspondence is seen to exist in the degree of inhibition effected. Nicotine inhibits fructose oxidation by brain (Table V) to an even greater extent than glucose oxidation, 50 per cent at 0.015 M and

TABLE VII  
*Rat Testes Plus Nicotine*

Experiment No.	Concentration of nicotine	Glucose, 0.02 M				Lactate, 0.02 M			
		$-Q_{O_2}$	r.q.	$Q_A$	Inhibition of $Q_{O_2}$	$-Q_{O_2}$	r.q.	$Q_A$	Inhibition of $Q_{O_2}$
	mole per l.				per cent				per cent
1	0	11.5	0.91	+6.8		16.2	0.86	-1.7	
	0.015	7.6	1.01	+5.4	34	8.6	0.87	+0.5	47
2	0	9.7	0.95	+6.4		12.1	0.76	+0.3	
	0.015	6.6	1.05	+7.6	33	8.6	0.83	+1.4	29
3	0	12.9	0.95	+6.8		16.8	0.89	-1.8	
	0.03	4.8	1.01	+5.4	63	4.2	0.80	+1.0	75
4	0	13.0	0.94	+6.5		17.8	0.87	-1.8	
	0.03	4.6	1.00	+5.4	65	3.9	1.00	-0.1	78
5	0	12.3	0.91	+7.7		17.3	0.86	-1.1	
	0.03	2.8	1.13	+5.4	78	3.6	0.79	+2.2	79
6	0	12.8	0.98	+5.4		17.1	0.84	-1.9	
	0.03	2.5	1.20	+3.7	81	3.1	0.78	+1.7	82

85 per cent at 0.03 M. (See Table I for comparable figures on the inhibition of glucose oxidation.) There was no significant effect on the r. q. (0.93 for normal as compared with 0.87 to 1.00 with nicotine, and, unlike results with glucose, there was no increase in acid formation.

Succinate oxidation (Table V) was, in general, little affected by nicotine, even at a concentration of 0.03 M. The results were irregular, but in no case was an inhibition of more than 20 per cent produced.

*Testes and Kidney*—Results obtained from experiments on the

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respiration of rat kidney and testes are presented in Tables VI and VII. Apparently kidney respiration is less sensitive to nicotine than is that of cerebral cortex, the inhibition of respiration with 0.03 M nicotine being less than that produced on brain. In further contrast with brain, glucose and lactate oxidations by kidney are inhibited to about the same extent, 39 and 30 per cent respectively. A few experiments are indicated in Table VI in which no substrate was added. With 0.03 M nicotine, the inhibition was 44 and 47 per cent, approximately the same as that produced in the presence of either glucose or lactate. With testes (Table VII) the same type of result was obtained as with kidney; with 0.015 M nicotine, glucose oxidation was inhibited 33 per cent and that of lactate, 38 per cent (average of two experiments); with 0.03 M nicotine, glucose oxidation was inhibited 72 per cent, and lactate 78 per cent (average of four experiments). From the experiments here presented, it can be seen that in kidney and testes, unlike results with cerebral cortex, glucose and lactate oxidations are inhibited to approximately the same extent.

### DISCUSSION

The results presented in this paper indicate that at least in brain tissue it is possible for glucose to be oxidized under conditions in which lactic acid oxidation is almost completely inhibited, and the amount of glucose which disappears agrees well with the amount which should have disappeared, as calculated from oxygen uptake at a respiratory quotient of unity. According to these observations, glucose is oxidized completely to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  through intermediates other than lactic acid. However, our results do not preclude the possibility that in the absence of nicotine part of the respiration may go through lactic acid, thus indicating at least two paths for the oxidation of glucose in brain tissue. It is of interest to mention at this time a few of the experiments reported in the literature with various tissues and dyestuffs in which glucose oxidation was accelerated to a considerably greater extent than lactate oxidation (Dickens (1934); Dodds and Greville (1934); Pourbaix (1934); Young (1937)), which may be interpreted as lending additional support to the concept that two paths for glucose oxidation exist.

It has been suggested by various workers (Toenissen and

Brinkman (1930); Peters (1936); Elliott and Schroeder (1934)) that pyruvate is an essential step in the oxidation of lactic acid. We have found that the oxidation not only of lactate but also of pyruvate is inhibited by nicotine. Since vitamin B<sub>1</sub> is, according to Peters, essential for the oxidation of pyruvic acid by brain tissue, it would appear that nicotine blocks the same path that is catalyzed by vitamin B<sub>1</sub>.

The experiments with nicotine reveal that the oxidation of carbohydrate in the brain involves other processes than, for example, in kidney and testes, for, in the latter tissues, nicotine has no differential inhibitory effect on glucose and lactate oxidations. Cerebral tissue also differs from other tissues in its unaltered ability to oxidize carbohydrate in the absence of insulin. Recently more evidence has been accumulating, revealing the ability of the body to oxidize limited but significant amounts of carbohydrate in the absence of insulin (Houssay (1937); Chambers (1938); Himwich, Fazekas, and Martin (1938)). The brain, then, has developed this ability to a greater extent than other tissues of the body. This point of view is further corroborated in the present communication, which discloses that the glucose disappearing in the diabetic brain can be accounted for quantitatively by oxidations.

*Glycolysis*—Despite the inability of cerebral tissue to oxidize lactic acid, *aerobic* lactic acid formation is stimulated by nicotine. This is especially interesting in view of the communication of Quastel and Wheatley (1937), according to which nicotine stimulates the *anaerobic glycolysis* of brain slices.

#### SUMMARY

1. The metabolism of slices of cerebral cortex of rats and of normal and depancreatized cats was studied in the Dixon-Keilin apparatus in the presence and absence of nicotine. In the presence of a concentration of nicotine which prevents the oxidation of lactic acid, it was shown quantitatively that the disappearance of glucose could be accounted for by the oxygen consumption (corrections being applied for glucose going to form lactic acid). Moreover, this mechanism of glucose oxidation does not require insulin.

2. Oxidations in the brain differ from those in testes and kidney,

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where nicotine inhibits glucose and lactic acid oxidation to the same extent.

3. Nicotine inhibits the oxidation by cerebral tissue of various normal substrates to different degrees; pyruvate and lactate are inhibited to about the same extent, and to a much greater degree than glucose; fructose is also inhibited more than glucose; succinate oxidation is practically unaffected.

4. The aerobic glycolysis of brain, but not of testes and kidney, is accelerated by nicotine.

5. Nicotinic acid has an even more marked differential inhibiting effect on glucose and lactic acid oxidations than has nicotine; nicotinic acid amide, on the other hand, has little effect.

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## PREPARATION OF $\beta$ -GLUCOSE

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There are three laboratory methods used for the preparation of  $\beta$ -glucose; namely, the pyridine process of Behrend (1), the ammonia-alcohol method of Levene (2), and the acetic acid method of Hudson and Dale (3). A fourth method for obtaining good yields of  $\beta$ -glucose results from a modification and extension of Tanret's (4) original procedure.

Tanret recommended nucleation with  $\beta$ -glucose and stirring a concentrated glucose solution in an oven at  $110^{\circ}$  for 8 hours, in which time crystallization and dehydration took place. Although this complete dehydration procedure gives good yields of  $\beta$ -glucose, caramelization occurs to a considerable extent. By subjecting the sugar sirup to less drastic treatment caramelization can be practically eliminated. In the modified method an 85 per cent glucose solution is evaporated over a period of 2 hours in a vacuum oven at  $100^{\circ}$  to a solid anhydrous mass of crystals. The resulting pure white sugar shows almost no evidence of caramelization and possesses a rotation<sup>1</sup> of  $+26.1^{\circ}$ , representing 93 per cent  $\beta$ -glucose content. The sugar is readily purified by recrystallization from alcohol.

In the neighborhood of  $100^{\circ}$  the  $\beta$  modification is the stable form and is, moreover, less soluble than the  $\alpha$ -glucose (5, 6). Newkirk (7) shows a phase diagram in which he depicts *m*-stable  $\beta$ -glucose in the vicinity of  $100^{\circ}$  and up to a temperature of  $114^{\circ}$ , at which point it becomes the stable form for glucose. A hot concentrated glucose sirup might then be expected to deposit crystals of pure  $\beta$ -glucose. Experiments show that such is the case and that good yields of  $\beta$ -glucose can be obtained by fractional crystallization from water solutions at  $100^{\circ}$ .

<sup>1</sup>All rotations are specific rotations taken with the D line of sodium light at  $25^{\circ}$ .

## EXPERIMENTAL

*Complete Dehydration Method*—200 gm. of anhydrous  $\alpha$ -glucose (cerelese) were added slowly with stirring to 23.5 gm. of hot water (to produce an 85 per cent solution) and the mixture heated with constant stirring on a boiling water bath and then on a hot-plate until a clear solution was obtained. The sirup was then poured into a shallow Pyrex tray of such size that the sirup filled the tray to a depth no greater than  $\frac{1}{2}$  inch. The tray was then placed in a vacuum oven at 100° and a vacuum of not less than 27 inches applied to the oven. After 30 minutes the sirup was nucleated with a few crystals of pure  $\beta$ -glucose, thoroughly stirred, and replaced in the vacuum oven at 27 inches vacuum and 100°. It is important that a temperature of at least 100° be maintained during the entire crystallization of the  $\beta$ -glucose to insure its production in good yields. This temperature refers to the sirup and was measured by a thermometer placed in the crystallizing tray. 90 minutes after seeding a solid anhydrous mass of crystals resulted. The rotation of this sugar was +26.1°. It was then dissolved in 200 cc. of water at 0°. After 30 seconds of vigorous stirring the solution was quickly filtered and 1 liter of absolute alcohol mixed with the filtrate. Seeding and stirring produced an immediate crystallization of  $\beta$ -glucose having a rotation of +21.8°. A second recrystallization from alcohol produced crystals rotating at +18.8°. The yield was 160 gm. For preservation of the  $\beta$  form all traces of moisture were removed by drying in a vacuum oven for 3 hours at 75°.

In this procedure good yields of  $\beta$ -glucose were obtained without nucleation when small runs were made (*i.e.* 50 gm.). However, in large preparations nucleation with pure  $\beta$ -glucose materially increased the yield of this form.

*Method of Fractional Crystallization from Water*—200 gm. of anhydrous  $\alpha$ -glucose were dissolved in water as described above and the clear sirup in a liter beaker was placed in a vacuum oven as recommended in the first procedure. In 30 minutes the sirup which was near the point of crystallization was nucleated with pure  $\beta$ -glucose. After 15 to 30 minutes longer in the oven the sirup had crystallized to a thick mush. At this point the crystals were quickly removed from the oven, stirred with an equal volume of boiling 90 per cent alcohol, and filtered through a previously

warmed Buchner funnel having a 200 mesh monel metal screen in place of filter paper. The crystals were washed free of sirup with hot 90 per cent alcohol and then further washed with absolute alcohol. After drying for 3 hours at  $75^{\circ}$  in a vacuum oven the crystals rotated at  $+38.5^{\circ}$ . The yield was 140 gm. After one recrystallization from alcohol the rotation was reduced to  $+25.6^{\circ}$ . A second recrystallization gave crystals rotating at  $+19.3^{\circ}$ .

#### DISCUSSION

In both procedures  $100^{\circ}$  is found to be the optimum temperature for  $\beta$ -glucose production. Higher temperatures result in extended caramelization, while lower temperatures give but poor yields of the  $\beta$  modification. Since almost all of the water is removed from the sirup during the first 15 minutes of drying, a more dilute sirup might be used at the start, in place of the 85 per cent sirup.

#### SUMMARY

Modification and extension of Tanret's original procedure for the preparation of  $\beta$ -glucose make possible a fourth laboratory method for obtaining this sugar in good yield.

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## THE AMINO ACID CONTENT OF COW AND CHIMPANZEE HAIR

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Interest in more exact knowledge of the chemical composition of the keratins of various types has been greatly stimulated by the results obtained by the use of the methods of x-ray analysis as a means of detecting and measuring regularities of the molecular patterns of the fibrous keratins (1). For formulation of such patterns, more complete analyses of the amino acid components of the keratins are essential. The most complete and more recent studies have been concerned with wool, the chief fibrous keratin of commercial importance.

Further interest in keratins has been aroused by the recently proposed characterization of a true keratin (2, 3) as a protein with a relatively constant molecular ratio (1:4:12) of the basic component amino acids, histidine, lysine, and arginine. The constancy of this ratio suggests the presence of some uniform nucleus in the molecules of keratins derived from various biological sources.

Another characteristic of the fibrous keratins is their high content of sulfur and the sulfur-containing amino acid, cystine. Keratinization of a fiber has been associated with the process of cystinization (4, 5). Factors, age and diet, which may influence the hair of young white rats, have been investigated previously in this laboratory (6, 7). Workers in the Japanese laboratories have also studied variations of the cystine content of hair, related to species, sex, and age (8, 9).

No recent study of cow hair was available and we had been much interested in the report by Hsieh (10), that only 1.5 to 2.5 per cent of cystine could be isolated from cow hair. Since 5 to 6 per

cent of cystine can be obtained from human hair in isolations in which no attempt at quantitative procedures is made, the findings of Hsieh suggested a content of cystine in cow hair much lower than that of other animal fibers. The analyses of Okuda and Katai (9) were not yet available when this study was undertaken. Through the helpful cooperation of Professor G. L. Huffman of the Department of Dairy Husbandry of Michigan State College, we were able to obtain for study samples of hair from cows of pure breed (Holstein), and of known age and nutritive history. It was thus possible to secure more complete data concerning the amino acids of cow hair and also to study the age factor in cows of the same breed and herd in relation to the cystine content of the hair.

It has been possible to study the composition of the hair of chimpanzees of known sex and age with especial reference to the content of cystine.

#### EXPERIMENTAL

For extraction of the hair prior to analysis the samples were suspended in distilled water for 12 hours at room temperature, dried on filter paper in the laboratory for 12 to 20 hours, and then extracted in a continuous extraction apparatus at 45-50° with the various solvents. The extraction was carried out with 95 per cent alcohol, chloroform, absolute alcohol, and ether in succession for periods of 48, 18, 24, and 12 hours respectively. After the final extraction, the samples were dried in a vacuum oven at 60° for 24 hours and stored in a vacuum desiccator for analysis. No alteration in the character of the fiber was observed as a result of the extraction.

Moisture and ash were determined by the usual procedure, total nitrogen by the micro-Kjeldahl method of Pregl.

Total sulfur was determined by two methods, the modified Denis-Benedict procedure as used in this laboratory (11) and the Parr bomb method. In view of the difficulties in the application of the Benedict or Denis method to the analysis of methionine or of proteins containing significant amounts of methionine (12), the Parr bomb method was used in parallel determinations with the modified Denis-Benedict method. Since the keratins contain little methionine, it was believed that the use of the modified

Denis-Benedict method would introduce little error. This assumption proved to be justified by the results of the analyses. No significant differences in the content of total sulfur of the keratins, as determined by the two methods, were apparent. Comparative unselected analyses of six consecutive samples of cow hair gave 3.76 and 3.73, 3.90 and 3.87, 3.76 and 3.65, 3.81 and 3.69, 3.80 and 3.75, and 3.67 and 3.79 per cent of sulfur by the modified Denis-Benedict and the Parr bomb methods respectively. The values presented in Tables I and IV are those obtained by the use of the Parr bomb method.

Cystine was determined by the Sullivan method, as modified by Rossouw and Wilken-Jorden (13), tyrosine and tryptophane by the methods of Folin and Marenzi (14), basic amino acids by the methods of Block (15), and dicarboxylic amino acids by the methods of Jones and Moeller (16).

Proline was determined as described by Bergmann (17), and glycine by the method of Bergmann and Fox (18). Since, at the time this work was undertaken, Bergmann and Fox had not yet made available the exact details of their procedure, we made use of the slight modification described by Block (19) generously placed at our disposal by him. No correction for the solubility of the double salt of glycine with potassium trioxalatochromiate (20) was made.

### *Cow Hair*

The total nitrogen content of the cow hair ranged from 14.64 to 16.00 per cent, but, in general, was of the same order of magnitude in all the samples of hair and did not appear to bear any relation to the age of the animal (Table I). Total sulfur content varied only slightly in the thirteen samples analyzed (range, 3.62 to 3.87 per cent) and could not be correlated with age (Table I).

Humins nitrogen varied from 1.3 to 6.7 per cent of the total nitrogen, the higher values being obtained in the analyses of the black hair. Amide nitrogen comprised from 9.1 to 16.6 per cent of the total nitrogen. Although there appeared to be a tendency for a lower amide nitrogen in the hair of the younger group of animals (range, 9.1 to 12.1 per cent) than in the hair of the older animals (range, 13.4 to 16.6 per cent), no attempt was made to correlate these values with the content of the dicarboxylic amino

acids, since the methods for the determination of this latter group are not entirely satisfactory, and since the amount of the individual samples available for analysis was limited.

The contents of tryptophane and of tyrosine of the hair were relatively constant and also bore no relation to the age of the animal (Table I). In the case of cystine, an entirely different picture was presented. The values ranged from 10.97 to 13.58 per cent. In the group of animals 3 to 4 months of age there was

TABLE I

*Analysis of Samples of Cow Hair (Holstein) from Animals of Different Ages*

Two separate samples of hair from Animals 188 and 190 were analyzed, the one consisting of white and the second consisting of black hair, as indicated in the table. All values are calculated on an ash-free, moisture-free basis. The letters W., B., and M. indicate white, black, and mixed (black and white) hair respectively.

Animal No.	Color	Age	Total N	Total S	Cystine	Cystine S of total S	Tyrosine	Tryptophane
		mos.	per cent	per cent	per cent	per cent	per cent	per cent
193	W.	3	15.32	3.73	10.97	79.2	3.33	1.33
191	"	3	14.64	3.87	11.45	78.5	3.13	1.40
190	"	3	15.20	3.65	11.46	83.8	3.51	1.47
190	B.	3	15.54	3.69	11.24	81.5	3.23	1.43
188	W.	4	15.65	3.75	11.46	81.6	3.29	1.36
188	B.	4	16.00	3.79	11.45	81.0	3.25	1.37
189	M.	4	15.58	3.63	11.65	85.8	3.30	1.30
15	B.	49	15.61	3.77	12.65	89.8	3.45	1.42
8	W.	61	15.51	3.62	13.42	98.8	3.19	1.40
5	"	61	15.12	3.75	13.58	96.6	3.21	1.28
6	"	83	15.55	3.77	13.26	93.5	3.13	1.33
3	"	86	15.67	3.66	13.43	98.2	3.41	1.42
4	B.	87	14.84	3.62	13.36	98.6	3.35	1.29

a relatively small variation in the cystine content (10.97 to 11.65 per cent). In the older group, 49 to 87 months of age, the cystine content was consistently higher (12.65 to 13.58 per cent), as shown in Table I. The values for the hair of the adult animals were definitely higher than those reported by Okuda and Katai (9) and the variation with age in our own analyses is much more marked. The hair of animals younger than 24 months was not examined by them. The results obtained furnish further proof

that in the production of adult hair keratinization is accompanied by cystinization (4-6). The values for the cystine content of cow hair are comparable to those for other fibrous keratins and demonstrate clearly that there is not the low content of cystine in cow hair suggested by the isolation studies of Hsieh (10).

The cystine content was consistently higher in the hair of the older group, but the total sulfur was relatively uniform and bore no relation to the age of the animal. In the younger group, cystine sulfur comprised from 79.2 to 85.8 per cent of the total sulfur (Table I), while, in the older group, total sulfur was almost completely accounted for as cystine sulfur in most cases. While we realize the limitations of cystine determinations, we believe that the differences in the percentage of the total sulfur present as cystine sulfur are clearly beyond the experimental error of the analytical procedures and suggest the presence, in the hair of young animals, of considerable amounts of sulfur in some compound other than cystine. Further evidence in support of this hypothesis is afforded by consideration of the values for labile sulfur obtained by the method of Zahnd and Clarke (21). Since the source of labile sulfur is believed to be cystine, variations in the cystine content of the keratins should be reflected in the analyses for labile sulfur. The amount of material available limited our determinations to the analyses of four samples, two in each age group. The white hair of Animal 191 and black hair of Animal 190 yielded 2.84 and 2.80 per cent respectively of their sulfur as labile sulfur, equivalent to 72.8 and 73.5 per cent of the total sulfur. The two samples of white hair of the older group, Animals 6 and 3, gave on analysis values of 3.29 and 3.39 per cent of labile sulfur or 86.8 and 91.2 per cent respectively of the total sulfur.

Basic amino acids were determined in three samples of hair, one from the younger age group and the others from the older group (Table II). The values obtained were practically identical in all three samples and are similar to those reported previously for other animal fibers (2, 3, 22). When the percentages of these basic amino acids are expressed as a molecular ratio, the result, 1:4:10, is comparable to the ratio, 1:4:12, proposed by Block and Vickery (2, 3) in their characterization of keratins.

The amount of material at our disposal did not permit analyses

of the individual samples of hair for their content of glutamic and aspartic acid. Two samples of mixed hair, one from the younger group (Animals 190, 193, and 191) and one from the older group (Animals 3, 4, and 5) were analyzed. Values of 2.7 and 3.0 per cent of aspartic acid were obtained for the mixed samples of the hair of the younger and older groups respectively and values of 12.1 and 11.4 per cent for glutamic acid. Hair from Animals 3 and 8 contained 10.56 and 10.02 per cent of glycine respectively, and hair from Animals 4, 8, and 189, 8.28, 8.18, and 8.22 per cent of proline respectively. The average percentages of glycine and proline in cow hair were 10.28 and 8.28 per cent. The relatively high content of glycine is similar to the high figure of 9.12 per cent reported by Buchtala for human hair (23).

TABLE II  
*Basic Amino Acids of Cow Hair*

All values are calculated on an ash-free, moisture-free basis. The letters W. and B. indicate white and black hair respectively.

Animal No.	Color	Age	Histidine	Lysine	Arginine	Molecular ratio, histidine to lysine to arginine
		<i>mos.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
193	W.	3	0.69	2.14	7.42	1:4:10
15	B.	49	0.67	1.87*	7.48	1:3:10
4	W.	87	0.68	2.10	7.44	1:4:10

\* Slight loss.

In Table III are assembled the averages of all the amino acid determinations of cow hair made in the present series and, for comparison, typical values for wool as presented by Astbury and Woods (24).

### *Chimpanzee Hair*

Our interest in the composition of chimpanzee hair was aroused by the known evolutionary relationships between the anthropoid apes and man. There is a closer resemblance in respect to certain metabolic processes between the anthropoid apes and man than between these apes and the lower mammals. Human hair is greater in its content of cystine than is any other similar animal fiber. Is the content of cystine of the hair of the chimpanzee

similarly high? Through the courtesy of Professor R. M. Yerkes of the Laboratory of Primate Biology of Yale University, we have secured specimens of hair from chimpanzees of known age and sex, from animals which have been under observation for prolonged periods of time in the colony at Orange Park in northern Florida (26). For comparison we present analyses of three samples of human hair also (Table IV).

TABLE III

*Amino Acid Content of Cow and Chimpanzee Hair and Sheep Wool*

The values for wool are those compiled by Astbury and Woods (24), except where noted.

Amino acid	Cow hair	Wool	Chimpanzee hair
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Histidine.....	0.67	0.6	0.64
Tryptophane.....	1.37	1.8	1.42
Lysine.....	2.03	2.8	2.02
Aspartic acid.....	2.86	7.3*	
Tyrosine.....	3.29	4.8	3.32
Arginine.....	7.45	10.2	8.09
Proline.....	8.28	4.4	
Glycine.....	10.28†	0.6	
Glutamic acid.....	11.76	15.3*	
Cystine.....	13.41‡	13.1	15.50§

\* Values obtained by Speakman and Townend (25).

† If the glycine value is corrected (20), a figure of 11.68 per cent is obtained.

‡ The values for cystine are averages of the analyses of the hair from the five animals whose age is greater than 60 months. The values for these animals are included because of the greater uniformity of the cystine content of the hair of the older animals.

§ Average of analyses of hair from three adult animals only.

The hair of the chimpanzee (Table IV) contained a higher percentage of sulfur and of cystine than cow hair, a percentage of sulfur only slightly less than that of the samples of human hair analyzed in the present study. There was observed a tendency toward a greater percentage of cystine of the hair of the older animals. Thus, the average cystine value for the hair of the two younger animals was 14.55 per cent and that of the samples of adult chimpanzee hair, 15.50 per cent. The percentage of cystine



in chimpanzee hair was similar to that in the three samples of human hair analyzed. The content of total sulfur did not vary but, as in the studies of cow hair, it was possible to account for a greater percentage of the total sulfur of the hair as cystine sulfur in the older animals (Table IV).

Tyrosine and tryptophane values were relatively uniform for all the samples of chimpanzee hair and were comparable to those already presented in our analyses of cow hair. It was possible to determine the basic amino acids in a single sample of chimpanzee hair only (Animal 4). The percentages of histidine, lysine, and

TABLE IV  
*Chemical Composition of Hair of Chimpanzee and of Man*  
All values are calculated on an ash-free, moisture-free basis.

	Animal No.	Sex	Age	Total N	Total S	Cystine	Cys-tine S of total S	Tyrosine	Tryp-to-phane
			yrs.	per cent	per cent	per cent	per cent	per cent	per cent
Chimpanzee	1	M.	1.5	16.54	4.37	14.42	88.0	3.16	1.44
	2	F.	3	16.62	4.25	14.67	92.1	3.16	1.41
	3	"	11	16.66	4.42	15.70	95.0	3.34	1.36
	4	M.	15	16.86	4.20	15.45	98.1	3.63	1.44
	5	F.	17	16.75	4.30	15.30	94.9	3.34	1.43
Human	6	" *	24	16.66	4.80	15.40	85.5	2.57	1.42
	7	M. †	24	16.57	4.83	15.45	85.6	3.31	1.27
	8	" ‡	70	15.52	5.08	15.93	83.6		

\* Blond hair.

† Red hair.

‡ Gray hair.

arginine recorded in the compilation of Table III are similar to those of cow hair. The molecular ratio of the basic amino acids is approximately 1:4:10; *i.e.*, chimpanzee hair is a keratin as defined by Block and Vickery (2).

Okuda and Katai (9) have reported analyses of the cystine content of the hair of various species of monkeys, but analyzed only one sample of hair from each species. No details as to sex or age are available. The cystine values (determined by the iodometric method of Okuda) are very variable, ranging from 10.18 (gibbon) to 14.48 (pig-tailed monkey) per cent. The values ob-

tained by us in our analyses of chimpanzee hair are uniformly high and suggest that the high content of cystine of the hair is a further point of similarity between man and the anthropoid apes.

#### SUMMARY

1. In order to afford more complete data concerning the composition of a fibrous keratin other than wool, a study of the composition of cow hair, which included analyses for ten of the amino acids, has been made.

2. The variations in composition associated with the age of the animal have been studied by the examination of thirteen samples of hair from pure bred Holstein cows of the same nutritive history, but varying in age from 3 to 87 months. The cystine content ranged from 10.97 to 11.65 per cent in the hair of younger animals (3 to 4 months) and from 13.26 to 13.58 per cent in the hair of adult cows (61 to 87 months).

3. The hair of the chimpanzee resembled human hair in the high content of total sulfur and cystine. The cystine content of the hair of older animals was slightly higher than that of the younger animals, but the total sulfur did not vary.

4. The molecular ratios of the three basic amino acids of both cow and chimpanzee hair were such as to characterize these fibrous proteins as keratins, as defined by Block and Vickery (2).

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# A SIMPLIFIED ESTIMATION OF LACTATE IN NORMAL HUMAN BLOOD

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We have been accustomed to estimate the lactate in blood by hemolysis in water, removal of the proteins by the method of Folin and Wu (4), treatment of the filtrate with  $\text{CuSO}_4$  and  $\text{Ca}(\text{OH})_2$  according to Van Slyke (11), and estimation of the lactate in the centrifugate by a modification of the aeration method of Friedemann, Cotonio, and Shaffer (6). There have been suggestions that some of the precautions usually taken are not necessary for filtrates from normal blood. For instance, several workers (2, 10) have confirmed the suggestion of Friedemann, Cotonio, and Shaffer (6) that treatment of Folin-Wu filtrates with  $\text{CuSO}_4$  and  $\text{Ca}(\text{OH})_2$  might be omitted in some cases. We have found that when normal human blood is used certain other customary steps have no effect on the estimated lactate, and may therefore be left out.

The apparatus<sup>1</sup> (Fig. 1) is a modified form of Wendel's (13). It takes up small space and is less liable to break than his original form. The glass joints are all interchangeable. Rubber stoppers are used at points where quick strains occur. The absorption towers are similar to Wendel's (13).

*Reagents*—These are all made according to Friedemann, Cotonio, and Shaffer (6) and Folin and Wu (4), except our sodium bisulfite, which is 5 per cent.

## Procedure

Heparinized blood, cells, and plasma, prepared from heparinized blood, and blood without anticoagulant added, are convenient to

\*Died December 14, 1937. This paper was finished by his colleagues, F. Consolazio and R. E. Johnson.

<sup>1</sup>Procurable from the Macalaster-Bicknell Company, Cambridge, Massachusetts.

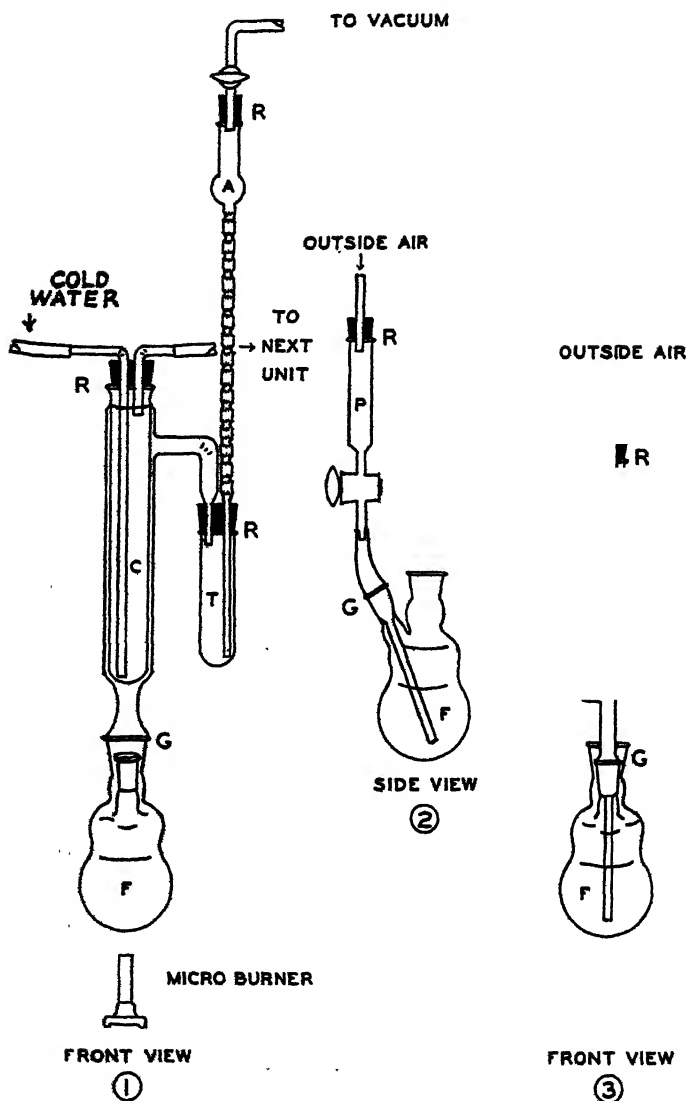


FIG. 1. Diagram of the apparatus to scale. The height of the reaction flask is 15 cm.; its capacity, 275 cc. A is the absorption tower, C the condenser, F the reaction flask, G the glass joints, P the permanganate reservoir, R the rubber stoppers, T the tube for bisulfite. When all are assembled, P fits in F, F on C, T on C, and A in T.

use. Pipette 1 cc. of whole blood into 45 cc. of distilled water; mix. Pipette 1 cc. of plasma into 17 cc. of distilled water; mix. Pipette 1 cc. of cells into 53 cc. of distilled water; mix. (For cells, use a pipette calibrated "to contain," and wash it out.) Add, mixing between additions, for whole blood 2 cc. of  $\frac{2}{3}$  N  $\text{H}_2\text{SO}_4$  and 2 cc. of 10 per cent tungstate. Add, mixing between additions, for plasma 1 cc. of  $\frac{2}{3}$  N  $\text{H}_2\text{SO}_4$  and 1 cc. of 10 per cent tungstate. Add, mixing between additions, for cells 3 cc. of  $\frac{2}{3}$  N  $\text{H}_2\text{SO}_4$  and 3 cc. of 10 per cent tungstate. Centrifuge 5 to 10 minutes at 3000 R.P.M. Turn on the water in the condensers.

To each reaction flask add 5 cc. of 10 N  $\text{H}_2\text{SO}_4$ , 10 cc. of 10 per cent  $\text{MnSO}_4$ , and about 50 cc. of distilled water. About ten subsequent runs can be made without adding new reagents or cleaning out the apparatus. Add fresh unknowns directly to the residues in the flasks, even when these are muddy brown, or hot, or both. Pipette aliquots of the centrifugate into the reaction vessels. Add 1 cc. of 5 per cent  $\text{NaHSO}_3$  to each tube. Adjust on the tower, and connect all rubber stoppers. Fill the reservoir with approximately 0.005 N  $\text{KMnO}_4$ . Adjust the air to flow at a brisk rate, light the burners, bring to the boiling point, and add  $\text{KMnO}_4$  by adjusting the stop-cocks to drip so that after 10 minutes a faint pink shows in each flask. About 1 drop every 10 to 15 seconds is needed. After the first run, a brown precipitate obscures the pink color, but add  $\text{KMnO}_4$  at the same rate as before. Add  $\text{KMnO}_4$  for a total of 15 minutes. Turn off the burners, shut off  $\text{KMnO}_4$ , and stop the flow of air. Wash down each tower into its corresponding tube with four 2 cc. portions of water, using a fast flowing pipette. Wash the tip of the tower with about 1 cc. Titrate as usual (6), avoiding a large excess of  $\text{I}_2$ . Use standard 0.005 N  $\text{I}_2$  and, for hydrolysis, about 5 gm. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  ground to a powder.

We have a battery of eight units. By using a duplicate set of tubes and titrating one set while another set of estimations is boiling, we can finish eight estimations every 25 minutes. This procedure has been worked out according to the conditions discussed in the body of the paper. We have used it with satisfaction in over 1500 routine estimations.

This method was definitively checked by comparing values obtained on the same blood with it and with the most specific of the

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methods for estimating lactate, that of Fletcher and Hopkins (3). For isolating the zinc lactate, the blood was deproteinized with 9 volumes of 95 per cent ethyl alcohol, aliquots of the filtrate were evaporated almost to dryness on the steam bath, and from that point the procedure of Fletcher and Hopkins was followed exactly, except that the aqueous solution of lactic acid prepared by ether extraction, before being heated with  $\text{ZnCO}_3$ , was treated with  $\text{PbO}$ , filtered, exposed to  $\text{H}_2\text{S}$ , filtered, and aerated (14). The two methods agreed well enough to suggest that by the present method principally lactate is determined in centrifugates from normal human blood (Experiment 1).

The three main steps of the estimation are deproteinizing the blood, oxidation of the lactate, and titration of the bound bisulfite. These steps will be considered in reverse order, because

*Experiment 1. Comparison of Lactate Determined by Present Method with That by Method of Fletcher and Hopkins*

Blood No.	Lactate in 100 cc. blood	
	Present method	Fletcher and Hopkins
	mg.	mg.
1	70.2	71.8
2	98.6	101
Recovery of standard lithium lactate, % of theory	96-102	89-95

the effect of variations in procedure at one stage can be more conclusively demonstrated if all further steps are properly verified. All of the blood used was drawn from the median cubital veins of normal, healthy men. High values were obtained by making the subjects run. All values for blood lactate are the averages of satisfactory duplicates or triplicates and are expressed as mg. of lactic acid in 100 cc. of blood. Every run contained one blank and one sample of standard lithium lactate. Acceptable runs had blanks of not more than 0.04 cc. of 0.005 N  $\text{I}_2$ , duplicates agreeing to 0.02 cc. of 0.005 N  $\text{I}_2$ , and a recovery of at least 96 per cent of theoretical on standard lithium lactate.

#### *Titration*

A small reagent blank is desirable. With good reagents (13) this should equal the distilled water blank. Sometimes a high

reagent blank is due to the tungstate, which, when boiled with a little dilute  $\text{H}_2\text{O}_2$ , then gives a low blank. Another cause of high blanks is excess of  $\text{I}_2$  during titration. In Experiment 2, 0.05 cc. of 0.25 N  $\text{I}_2$  was added in excess of the amount necessary to remove the excess  $\text{NaHSO}_3$  during titration, and various times were allowed to elapse before the excess  $\text{I}_2$  was removed with dilute  $\text{NaHSO}_3$ . There is no need to hurry with the titration at this point, but if excess  $\text{I}_2$  is present, a higher blank is found.

### *Oxidation*

Friedemann and Kendall (8) and Friedemann and Graeser (7) abandoned aeration from  $\text{H}_2\text{SO}_4$  in favor of distillation from  $\text{H}_3\text{PO}_4$  because, although all methods give good recovery with synthetic lactic acid, the distillation method gives the lowest

*Experiment 2. Effect of Excess Strong  $\text{I}_2$  during Titration*

Excess 0.25 N $\text{I}_2$ added	Period of exposure	Lactic acid added				Lactic acid recovered			
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
cc.	sec.								
0	0	0.290	Blank	0.180	Blank	0.280	0.004	0.180	0.004
0.05	10	0.290	"	*	*	0.282	0.007	*	*
0.05	30	0.290	"	0.180	Blank	0.281	0.009	0.178	0.011
0.05	60	*	*	0.180	"	*	*	0.177	0.011

\* No determination.

estimates with many types of biological materials and is therefore more specific than other methods. However, their (7) figures conclusively show that with copper lime-treated filtrates from normal human, sheep, dog, and rabbit blood there is no difference between results with the aeration and distillation methods. Under our conditions there is also no difference among various concentrations of  $\text{H}_2\text{SO}_4$  and  $\text{H}_3\text{PO}_4$ . Folin-Wu filtrates were prepared from several bloods. Some of each filtrate was treated with copper lime. The effect of preliminary aeration for 5 minutes was tested at the same time.

Experiment 3 demonstrates that with normal blood, copper lime treatment does not affect the estimated lactate, and that all of the concentrations of acid recommended by Friedemann *et al.* (6-8) give the same result. From considerations detailed below,



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N  $\text{H}_2\text{SO}_4$ . Friedemann (5) showed that copper lime treatment of glucose solutions does not affect the recovery of lactate, and Boyland (1) confirmed this. Friedemann, Cotonio, and Shaffer (6) pointed out that copper lime may not always be necessary for blood. Table V in the paper of Friedemann and Graeser (7) indicates that this is true, and Cook and Hurst (2) and Scott and Berg (10) have confirmed it for tungstate filtrates.

*Experiment 3. Effect of Copper Lime, Preliminary Aeration, and Various Concentrations of  $\text{H}_2\text{PO}_4$  and  $\text{H}_2\text{SO}_4$  on Recovery of Lactate from Blood Centrifugates*

Blood No.	Treatment	Estimated lactic acid in 100 cc. blood			
		No copper lime		Copper lime	
		No aeration	Aeration 5 min.	No aeration	Aeration 5 min.
		mg.	mg.	mg.	mg.
1	2% $\text{MnSO}_4$ , N $\text{H}_2\text{SO}_4$	20.8	21.2	21.0	22.9
	2% " 0.1 N "	23.4	23.9	22.8	21.7
	2% " 0.06 " $\text{H}_2\text{PO}_4$	24.0	24.0	22.3	23.5
2	2% " N $\text{H}_2\text{SO}_4$	126	127	129	128
	2% " 0.1 N "	126	128	127	131
	2% " 0.06 " $\text{H}_2\text{PO}_4$	128	134	127	133
3	2% " N $\text{H}_2\text{SO}_4$	73.6	73.0	70.6	71.1
	2% " 0.1 N "	73.6	72.4	73.9	72.0
	2% " 0.06 " $\text{H}_2\text{PO}_4$	72.5	73.1	74.3	73.4
3	With added lithium lactate equivalent to 90.0 mg. lactic acid in 100 cc. blood				
	2% $\text{MnSO}_4$ , N $\text{H}_2\text{SO}_4$	164	162	161	161
	2% " 0.1 N "	165	162	163	163
	2% " 0.06 " $\text{H}_2\text{PO}_4$	163	162	163	164

A further simplification emerges from Experiment 3. Since the amount of volatile bisulfite-binding substances in normal blood is negligible in comparison even to the blood lactate in resting subjects, preliminary aeration before oxidation is unnecessary.

Cook and Hurst (2), using Folin-Wu filtrates and trichloroacetic acid filtrates of human blood, showed that copper lime has no effect on the estimated lactate in tungstate filtrates, but lowers it in trichloroacetic acid filtrates. Their data, however,

did not directly compare the two types of deproteinization on the same blood. In Experiment 4 such a comparison is made with and without copper lime and with and without preliminary aeration. As explained below, we use a 1:50 instead of a 1:10 dilution of blood in our tungstate precipitation. With both trichloroacetic acid and tungstate, we got the same estimate of lactate under all conditions. The discrepancy between our results and those of Cook and Hurst (2) may arise because we use an aeration method, they a distillation. Friedemann and Graesser's Table V (7) suggests that distillation gives higher values than aeration on Folin-Wu filtrates not treated with copper lime, but the same

*Experiment 4. Effect of Copper Lime and Preliminary Aeration on Estimated Lactate in Folin-Wu Centrifugates and in Trichloroacetic Acid Centrifugates of Same Blood*

The results are expressed as mg. of lactic acid per 100 cc. of blood.

Blood No.	Blood diluted 1:50 in 0.2 per cent tungstate				Blood diluted 1:10 in 4 per cent trichloroacetic acid			
	No preliminary aeration		5 min. preliminary aeration		No preliminary aeration		5 min. preliminary aeration	
	No copper lime	Copper lime	No copper lime	Copper lime	No copper lime	Copper lime	No copper lime	Copper lime
1	39.4	39.4	37.2	41.1	40.8	41.1	42.2	41.4
2	22.7	23.8	23.4	23.4	23.4	18.6*	25.2	23.0
3	73.6	70.6	73.0	71.1	73.6	73.4	73.4	72.9

\* Error in diluting suspected.

result with copper lime-treated filtrates, and further, that aeration gives the same result with or without copper lime.

One of the time-wasting features of routine lactate estimations is cleaning the apparatus and adding fresh  $\text{MnSO}_4\text{-H}_2\text{SO}_4$  mixtures between runs. If fresh mixtures are not prepared after every run, but known lactate solutions or blood filtrates are added directly to the old solutions, and the estimation is carried out as usual, results are still quantitative. Experiment 5 was carried out without changing  $\text{MnSO}_4\text{-H}_2\text{SO}_4$  solutions after any of the runs.

In our experience the only limit to the use of old solutions is the volume of the reaction vessel. Dilution has no effect on the quantitative recovery of lactate, unless very dilute acid is used

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at the beginning. We have made as many as twelve runs with the same  $\text{MnSO}_4\text{-H}_2\text{SO}_4$  solutions, the last on lithium lactate (Experiment 6).

The solutions were a deep brown, with a muddy precipitate, but results were good. It is not even necessary to allow the solutions to cool down between runs. If preliminary aeration has to be performed, solutions must be changed as soon as a perceptible precipitate of  $\text{MnO}_2$  appears; otherwise, some lactate is destroyed during the preliminary aeration.

### *Experiment 5. Effect of Not Changing $\text{MnSO}_4\text{-H}_2\text{SO}_4$ Mixture between Runs*

The results are expressed as mg. of lactic acid per 100 cc. of blood.

Run No.	Blood 1	Blood 2	Lithium lactate containing 90.0 mg. lactic acid in 100 cc.	Mixture in flask
1	70.6	88.2	89.4	cc.
2	71.3	Another blood filtrate used in the apparatus	90.4	50 Before
3	71.0	" "	89.1	
4	72.1	" "	Lost	
5	72.4	91.4	90.0	186 After

### *Experiment 6. Recovery of Lithium Lactate after Eleven Runs without Cleaning Flasks*

The results are expressed as mg. of lactic acid.

Flask No.....	1	2	3	4
Added.....	Blank	0.145	0.145	0.145
Recovered.....	0.002	0.148	0.146	0.146

We do not use colloidal  $\text{MnO}_2$  instead of dilute  $\text{KMnO}_4$  (7, 8), because, under our conditions, the two oxidizing agents give the same estimate on the same centrifugate, and  $\text{KMnO}_4$  is the more convenient. The high concentration of  $\text{MnSO}_4$  we use follows the practice of Friedemann and Kendall (8). We adopted Wendel's (13) method of adding  $\text{KMnO}_4$  by allowing the reservoirs to drip slowly throughout the estimation.

With some pathological bloods preliminary aeration, copper

lime treatment, or both, may be necessary to give satisfactory lactate estimates. Experiment 7 gives the results with two types of pathological blood.

Copper lime should not remove alcohol, but preliminary aeration should drive it off. Van Slyke (11, 12) has shown that copper lime does not remove acetone bodies from urine, and one would not expect it to do so from blood centrifugates, but with our cases of ketosis not only copper lime, but also preliminary aeration had no effect. This was probably because acetone is so feebly bound by bisulfite (6). The necessity for these treatments will have to be determined for each type of pathological blood.

*Experiment 7. Effect of Preliminary Aeration and Copper Lime on Estimated Lactate in Pathological Blood*

The results are expressed as mg. of lactic acid per 100 cc. of blood.

Description of patient	No copper lime		Copper lime	
	No aeration	5 min. aeration	No aeration	5 min. aeration
Acute alcoholic coma.....	51.3	21.2	54.1	20.6
Mild ketosis (blood acetone, expressed as acetone, 6.0 mg. %)..	15.6	16.0	14.5	13.4
Mild ketosis (blood acetone, expressed as acetone, 15.0 mg. %).	32.3	31.8	33.4	30.1

*Preparation of Filtrates*

In the usual Folin-Wu procedure (4), a dilution of whole blood in the ratio 1:10 is used, and aliquot parts of the filtrate are taken on the assumption that lactate is distributed in the same ratio by volume throughout both the filtrate and the precipitate. If this assumption were true, the same estimate of lactate should be obtained whatever the final dilution. Fig. 2 is drawn from experiments, not on the same blood sample, in which 1 cc. samples of whole blood, plasma, or cells was added to various amounts of water and then, with mixing between additions,  $\frac{2}{3}$  N H<sub>2</sub>SO<sub>4</sub> and 10 per cent sodium tungstate were added. The mixtures were centrifuged, and aliquots of the centrifugates were analyzed. Since in the higher dilutions the usual concentrations of the Folin-Wu reagent no longer precipitated all the proteins, in every

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dilution the following amounts were used. Enough water was used to give the final dilutions indicated in Fig. 2.

1 cc. of each	$\frac{1}{2}$ N $\text{H}_2\text{SO}_4$	10 per cent tungstate
	cc.	cc.
Whole blood.....	2	2
Cells.....	3	3
Plasma.....	1	1

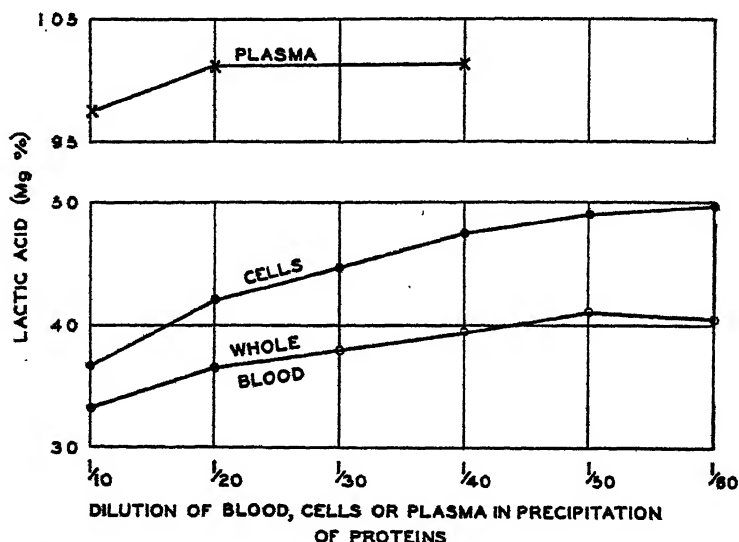


FIG. 2. Effect of increasing dilution with water on estimated lactate in whole blood, cells, and plasma, when deproteinized with  $\text{Na}_2\text{WO}_4$  and  $\text{H}_2\text{SO}_4$ .

The different amounts of protein in whole blood, plasma, and cells probably made it necessary to alter the usual concentrations of tungstate and acid in order to precipitate proteins properly in these higher dilutions.

If maximal estimates are taken to be correct estimates, then the following dilutions should be used for uniform results: for whole blood 1:50, for cells 1:60, and for plasma 1:20. We have done some experiments with whole blood in which a 1:10 dilution gave the same result as 1:50, but we do not understand the cause of these variations from experiment to experiment. In all of our

experiments, however, a 1:50 dilution has given maximal estimates, and we use this dilution routinely. It has the further advantage of giving a large volume of filtrate with consequent smaller percentage error in pipetting. Ronzoni and Wallen-Lawrence (9) have shown this dilution effect both for tungstate and for other types of protein precipitant. They found that a 1:10 dilution of blood in 0.2 per cent tungstate gives larger estimates than a 1:5 dilution. We are at present trying to determine what factors influence this phenomenon. Ronzoni and Wallen-Lawrence (9) postulated that lactate combines with the protein precipitate. It may be that variations in the quality of the tungstate give variations in the dilution effect (4).

The assumption that maximal estimates are correct estimates is supported by four kinds of evidence. First, the usual trichloroacetic acid centrifugate always gives the same value as a 1:50

*Experiment 8. Agreement between Calculated and Observed Cell Lactates*

The results are expressed as mg. of lactic acid per 100 cc. of blood.

Blood No.	Whole blood lactate	Plasma lactate	Cell lactate	
			Observed	Calculated
1	71.0	109	26.5	26.5
2	101	131	60.9	63.4

Folin-Wu centrifugate, but usually not the same as a 1:10 centrifugate (Experiment 4). Secondly, it is usually impossible to obtain agreement in calculated and observed cell lactate with the 1:10 Folin-Wu precipitation, but with the concentrations recommended above observed and calculated cell lactates always agree very well. Experiment 8 is an example.

Thirdly, if the same blood sample is divided in two equal parts, and if one part is precipitated in a 1:50 dilution and estimates are made on aliquots of the centrifugate, these estimates agree (Experiment 9) with those obtained from the other part by quantitatively extracting the blood four times with dilute tungstate-sulfuric acid mixtures, and pooling all the washings. Finally, the results obtained from the tungstate centrifugates agree tolerably well with those obtained by the isolation of zinc lactate according to the method of Fletcher and Hopkins (Experiment 1).

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the blood before analyzing it. To check this point, blood samples at all stages of deproteinization were allowed to stand without

### *Experiment 9. Comparison between Aliquots of Centrifugate and Quantitative Extraction of Same Blood Sample*

The results are expressed as mg. of lactic acid per 100 cc. of blood.

Blood No.	Value estimated from aliquot	Value estimated from extract
1	70.0	72.5
2	77.2	75.3
3	71.9	69.7

### *Experiment 10. Stability of Blood at Various Stages of Precipitation*

The results are expressed as mg. of lactic acid per 100 cc. of blood.

Tem- pera- ture...	Hemolysate				Hemolysate pptd. not centrifuged				Centrifugate			
	Ice box		Room		Ice box		Room		Ice box	Room		
	1	2	7	6	1	2	3	6		4	5	8
Blood No....												

Value when drawn

63.4 | 42.4 | 95.9 | 12.3 | 63.4 | 42.4 | 70.2 | 12.3 | 70.2 | 99.2 | 97.0 | 113

Value after standing

days												
1	62.4	41.0	98.6		61.7	43.6						
2	66.2	43.2	99.6		62.8	41.6						
3	63.2	42.7	94.4		62.0	44.6						
4		43.1	69.0		62.1	43.8						
5		44.3			62.4	44.3	72.4	12.6	71.7			
7								11.3				
11				11.3				14.5		99.0		
12							49.1					
13												112
16							32.0					
21											99.4	

aseptic precautions in the ice box and also at room temperature (around 24°). They were analyzed at suitable intervals after preparation (Experiment 10).

In order to inhibit the relatively strong glycolytic system of whole blood, hemolysis is advisable as soon as possible after the blood has been drawn.

The various preparations were quite stable, even at room temperature (about 24°), although eventually the values dropped in the case of hemolysates and hemolysates precipitated but not centrifuged. At ice box temperatures (about 4°) even the hemolysates seem to be stable for considerable periods. The centrifugates were remarkably stable at room temperature. There is, therefore, no need for analyzing the blood very quickly after it has been drawn, provided that it is at least hemolyzed. These findings agree with those of Ronzoni and Wallen-Lawrence (9) on the stability of tungstate filtrates from blood.

#### SUMMARY

1. A simplified method is described for estimating lactate in normal human blood.

2. Use of  $\text{CuSO}_4\text{-Ca(OH)}_2$ , preliminary aeration, and cleaning of the apparatus between runs are shown to be unnecessary.

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# MONOLAYERS OF DENATURED EGG ALBUMIN

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Within recent years there has accumulated a considerable literature dealing with the spreading of native proteins in monolayers. All attempts, however, to spread denatured proteins have been unsuccessful except those of Gorter (1), who treated heat-denatured egg albumin with pepsin and produced spreading. The purpose of the present paper is to report a simple technique by which urea-denatured and heat-denatured egg albumin can be spread in monolayers. The properties of these monolayers have been studied by means of a Langmuir surface balance and compared with those of monolayers of spread native protein. The hydrophilic character of the spread native protein has been quantitatively investigated and related to the structural changes occurring in the film as the film pressure is increased. The surface coagulation of heat-denatured protein is also described.

## EXPERIMENTAL

The egg albumin used in these experiments was prepared from fresh hen's eggs according to the method of Kekwick and Cannan (2). After four recrystallizations with sodium sulfate, the protein was dialyzed and then electrodialed to a specific conductivity of  $1.93 \times 10^{-5}$  reciprocal ohms. The solution at this point had a pH of 4.72, was water-clear, and the protein was 97.8 per cent heat-denaturable. It was preserved with a half and half mixture of washed toluene and mineral oil and stored at 2°.

A Langmuir surface balance manufactured by the Central Scientific Company under the trade name of the Cenco Hydrophile balance was used in all spreading experiments. Before each determination the aluminum tray was cleaned with boiling water

and scrubbed with a cloth. A solution of paraffin wax in benzene was applied to the hot, dry surface of the tray with a brush and allowed to dry and harden.

Experiments were conducted at room temperature which varied between 24–25°. N/150 sodium acetate buffer was used in the tray at a pH of  $4.90 \pm 0.05$ . All pH determinations were made with a Coleman glass electrode. The acetate buffer was always discarded after 5 hours of use in the tray.

Before each experiment the surface of the buffer in the tray was swept clean with the movable, paraffined barrier, and the balance with the mica float set in place. 0.325 cc. of the protein solution was then spread on the surface of the buffer from a graduated, calibrated, 1 cc. pipette. This pipette was clamped vertically over the surface of the buffer, dipped into the surface, and then slightly withdrawn, so that a cone of liquid formed around the tip of the pipette.<sup>1</sup> The protein solution was forced out gradually by means of a Hoffman screw clamp fitted on an eye dropper bulb attached to the top of the pipette. The concentration of all spread protein solutions was 0.00887 per cent. 15 minutes were allowed for spreading after the pipette was emptied.

Heat-denatured protein was obtained by placing 1.195 cc. of 0.742 per cent native protein solution in a 50 cc. Erlenmeyer flask, adding 20 cc. water and the desired quantity of 0.1 N HCl, and immersing the flask in a boiling water bath for 10 minutes. The flask was then quickly cooled under running tap water and the mixture made up to volume in a 100 cc. volumetric flask. All pH determinations reported on the spread solutions were made on these final, diluted solutions.

Urea denaturation was accomplished by mixing 1.195 cc. of 0.742 per cent native protein solution with 2.0 cc. of 50 per cent urea solution and allowing the mixture to stand at 2° for 1 week. After this time 20 cc. of water were added, followed by the desired quantity of 0.1 N HCl, and the mixture was made up to volume in a 100 cc. volumetric flask. Again all pH values of spread solutions apply to the pH of these final, diluted solutions.

The native protein was spread from an 0.00887 per cent solution made up in a N/150 acetate buffer.

<sup>1</sup> Dr. D. R. Briggs of the University of Minnesota suggested this spreading technique to me.

The hydrophilic properties of the spread native protein were studied by means of contact angle experiments. While these experiments are not directly concerned with the problem of the spreading of denatured protein, they throw some light on the structure of spread films in general. The adhesion tensions reported were calculated from the contact angles by means of the equation

$$\text{Adhesion tension} = \sigma(1 + \cos\alpha)$$

where  $\sigma$  is the surface tension of water and assigned a value of 72 dynes per cm. and  $\alpha$  is the contact angle. The contact angles were the angles which a standard sized drop of water made with the protein film deposited on a thin microscope cover-glass. A contact angle of zero or an adhesion tension of 144 ergs per sq. cm. indicates complete wetting, while a contact angle of  $180^\circ$  gives zero adhesion tension and complete absence of any tendency to wet.

The technique of deposition of these films and of measurement of the contact angles was as follows: The cover-glasses were attached to silk threads and immersed vertically in the buffer in the tray through a clean surface. The native protein was then spread on the surface of the buffer, and the film pressure adjusted by moving the movable barrier. After each pressure adjustment the slides were slowly and uniformly withdrawn through the surface film until the entire range of pressures had been explored. Deposition of the film on the slide was assured by the low angle of contact that the film made with the slide and also by a decrease in the area of the film on the buffer surface as the slide was withdrawn. The deposited films were allowed to dry at room temperature for 2 hours. A standard sized drop of water was then placed on the deposited film and the slide immediately placed in a lantern projector and projected on a screen. The contour of the drop was drawn and the angles of contact measured for both sides. The two adhesion tensions for the two angles were averaged, and these averages are reported. As the projected drop was at least 10 cm. in diameter, the angles could be measured with ease.

The contact angles and the adhesion tensions calculated therefrom are manifestly not equilibrium values and are of significance only on a comparative basis. Experiment showed that the contact angles progressively decreased as the drop remained on the de-

posited film. The adhesion tension-film pressure curve (Fig. 4), however, maintained its original contour over a number of hours; the whole curve shifted to higher adhesion tension values with the passage of time.

It will be remembered that surface-denatured protein, produced by shaking native protein solutions at pH 3, occurs partly in a coagulated condition (3), and if the shaking is continued long enough, the surface-denatured protein will completely coagulate. This is in sharp contrast with the behavior of a solution of heat-denatured protein at this pH, which will not coagulate however long the solution may be heated. Since it has been found possible to spread heat-denatured protein, it was considered worth while to extend this analogy to surface denaturation still another step and to attempt to surface-coagulate a clear solution of heat-denatured protein at pH 3.0. Accordingly, 100 cc. of a 0.495 per cent native protein solution containing the desired quantity of HCl were put in an Erlenmeyer flask and heated in a boiling water bath for 10 minutes and cooled. The solution was entirely clear. It was divided into four 25 cc. portions, one of which was retained as a standard for the dipping refractometric analysis and for a pH determination. The other three portions were placed in 220 cc. glass-stoppered bottles, and nine marbles 1.5 cm. in diameter were added to each bottle. The stoppers were put in place and sealed with melted paraffin wax. The bottles were then clamped on the circumference of a wheel 25 cm. in diameter and rotated around a horizontal axis at 108 revolutions per minute. One bottle was removed at the end of each hour and the contents filtered. The clear filtrate was analyzed with a dipping refractometer (4), and the found concentration subtracted from the original concentration to obtain the amount coagulated.

### Results

Fig. 1 shows the pressure in dynes per cm. plotted against the area per molecule of protein expressed in sq. Ångström units, the molecular area being calculated on the assumption that the molecular weight of egg albumin is 40,500 (5). The curves for the urea- and heat-denatured protein were selected from the experiments which gave the largest spread areas. This happened to be the urea-denatured solution with a pH of 2.23 and the heat-

denatured solution with a pH of 2.27. These results were obtained by allowing ample time for adjustment of the film as the area was decreased. For example, the native protein curve required 5 hours to complete, 15 to 20 minutes pause being allowed between readings at the higher pressures.

If the linear portion of the pressure-area curves be extrapolated to zero pressure, an area is obtained which allows the various

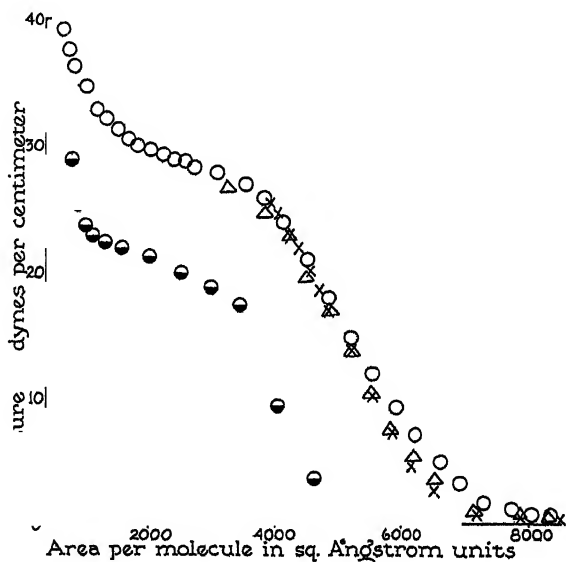


FIG. 1. Film pressure in dynes per cm. plotted against area in sq. Ångström units per protein molecule.  $\Delta$  curve for heat-denatured protein spread from a solution of pH 2.27,  $\times$  curve for urea-denatured protein spread from a solution of pH 2.23,  $\circ$  curve for spread native protein undergoing compression,  $\bullet$  curve for spread native protein undergoing expansion.

curves to be compared. This extrapolation is without theoretical significance and serves simply as a convenient method of comparison. Fig. 2 shows the plot of such an extrapolation of all the curves of heat- and urea-denatured protein as a function of the pH of the spread solutions (the pH of buffer in the tray was always  $4.90 \pm 0.05$ ). The point for the heat-denatured protein at pH 4.40 is an approximation, since it was not possible to obtain

## 590 Monolayers of Denatured Egg Albumin

an extensive pressure-area curve for this very incompletely spread film. The lack of spreading at pH 4.40 of heat-denatured protein is in agreement with the results of Neurath (6) who was also unable to obtain spreading at this pH. The areas indicated in Fig. 2 are expressed in sq.m. per mg. of protein. The area of the spread native protein with a value of 1.04 sq.m. per mg. compares favorably with that of 1.06 found by Philippi (7) and others. The maximum spread area for the heat-denatured protein was 0.990 sq.m. per mg. and that for the urea-denatured protein was 0.987 sq.m. per mg. The maximum values were obtained from the curves shown in Fig. 1. The coefficient of compressibility of all

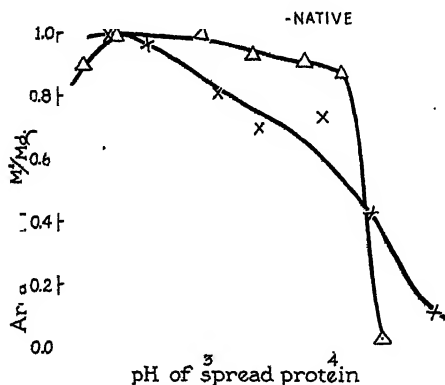


FIG. 2. Area of spread films in sq.m. per mg. as a function of pH of spread solution.  $\Delta$  heat-denatured protein,  $\times$  urea-denatured protein.

the native, heat- and urea-denatured protein films was calculated<sup>2</sup> from the linear portion of the pressure-area curves. A comparison of the compressibility of the films of native and heat-denatured protein with the pH of the spread solutions as well as with the extent of spreading, as shown by the extrapolated areas, failed to disclose any relation between these variables. The compressibilities of the native protein films (twelve values) were, therefore, averaged, and the probable error of the mean calculated; this was likewise done for the heat-denatured protein (ten values). The

<sup>2</sup> The coefficient of compressibility is defined as  $-(1/A_0) (dA/dF)$ , where  $A_0$  is the area of the film extrapolated to zero pressure, and  $dA/dF$  is the reciprocal of the slope of the linear portion of the pressure-area curve.

averaged compressibilities along with the probable error of the means are  $0.0157 \pm 0.0002$  cm. per dyne for the native protein and  $0.0160 \pm 0.000206$  cm. per dyne for the heat-denatured protein. The difference in compressibility of the two films is not significant.

The compressibility of the urea-denatured film showed a consistent variation with the extrapolated area of the spread film. This relation is shown in Fig. 3.

Fig. 4 shows the adhesion tension of water for the deposited film of spread native protein as a function of the pressure on the film at deposition. As emphasized in the experimental section, the calculated adhesion tensions are not equilibrium values, and

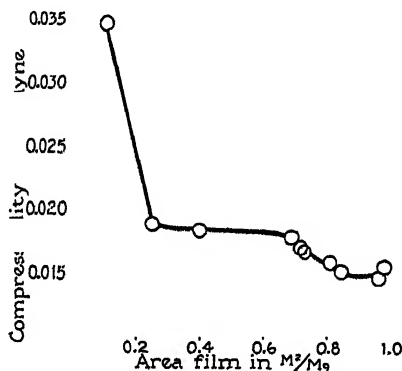


FIG. 3. Compressibilities of urea-denatured film in cm. per dyne as a function of spread areas of film.

subsequent checks (the curve was repeated twice) failed to show agreement in absolute amounts, but the shapes of the curves were in very satisfactory accord, and all the various inflections were found to occur at the same film pressures, as indicated in Fig. 4.

Fig. 5 shows the extent of surface coagulation of a clear solution of heat-denatured protein shaken at pH 3 as a function of time of shaking. The relation is a linear one and indicates that the rate of reaction is independent of concentration and is of a zero order. Such a rate of reaction is characteristic of surface reactions when the surface is saturated with the reacting materials. The coagulated protein formed a dense suspension of rather large aggregates which could be easily separated from the solution by filtration.



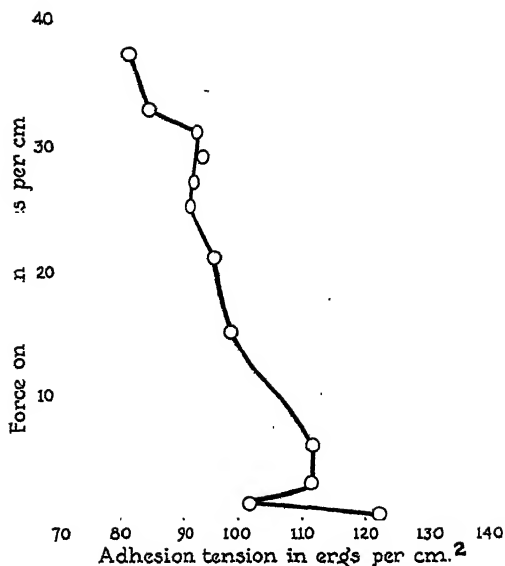


FIG. 4. Adhesion tensions of deposited films of spread native protein for water as a function of film pressure at deposition.

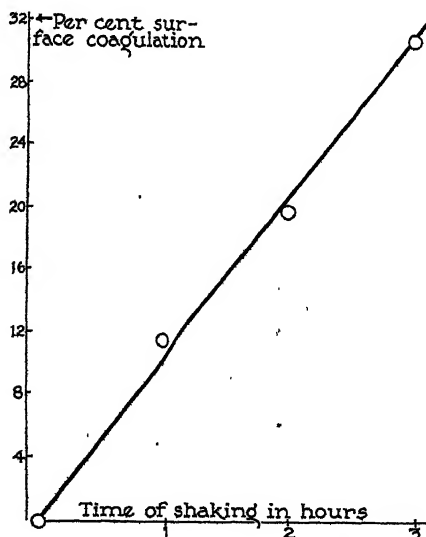


FIG. 5. Per cent of surface coagulation of heat-denatured protein produced by shaking a clear 0.495 per cent solution of heat-denatured protein at pH 3.0 as a function of time of shaking.

## DISCUSSION

It has been clearly demonstrated that heat- and urea-denatured protein can be spread in monolayers, provided the spreading solutions are adjusted to the proper pH. Since a denatured protein molecule fulfils the requirement for spreading, it must possess a polar character. Furthermore, the area and compressibility of such films closely approximate those for spread native proteins, indicating the probability of similar structure for these three completely spread forms (see Fig. 1). Neurath (6) has described the spreading of native protein as an unfolding of the peptide chain on the surface. It is tempting to identify the spread native protein with surface-denatured protein produced by the shaking of a native protein solution and to postulate an unfolded, asymmetric structure common to the surface-, heat-, and urea-denatured protein. There is much to be said for this simplifying assumption, both for the heat- and urea-denatured protein. For example, Williams and Watson (8) report the molecular weight of urea-denatured egg albumin to be 21,000 as determined by the ultracentrifuge, assuming a spherical particle. Burk and Greenberg (9) found the molecular weight of urea-denatured egg albumin to be 36,000 as determined by osmotic pressure measurements. The most plausible explanation for this divergence between the ultracentrifuge and osmotic pressure measurements is to assume a marked asymmetry produced in the native protein upon denaturation.

The situation is similar with the heat-denatured egg albumin. Loughlin (10), as well as other workers, found the viscosity of the heat-denatured egg albumin to be larger than that of the native protein at the same pH and protein concentration. So far as the author is aware, there are only three possible explanations for such an increase; namely, (1) association of particles, (2) increased hydration, and (3) increased asymmetry of the particle. The viscosity of the heat-denatured as well as that of the native protein solution is very nearly independent of pH, which would throw doubt on the first explanation, since it would be expected that association of particles would be greatly affected by pH changes. The hydration of heat-denatured protein is less than that of native (11), which would rule out the second suggestion. It is more reasonable to believe that the increase in viscosity is due to the

production of an asymmetric molecule from the symmetrical, native molecule upon heat denaturation. Using the equation of Guth (12) for the viscosity of a suspension of disks and substituting the concentration-viscosity data for heat-denatured egg albumin, we obtain a ratio of the long axis of the molecular disk to that of the short axis of 20.6. The same data substituted in the equation of Kuhn (13) for the viscosity of a suspension of rods gives a ratio of length to diameter of 11.1. While it is not possible to decide on the exact molecular structure from such data, these results do show the heat-denatured molecule to possess a very asymmetric character.

The idea of the unfolding of the molecule upon denaturation is also in accord with the observations of Mirsky and Anson (14) that the disulfide and sulfhydryl groups of egg albumin, which are undetectable in the native protein, are completely exposed in the denatured protein; an unfolding of the polypeptide chain would be expected to expose all the sulfur groups.

The hydrophilic properties should reflect the structural changes in the film as it is compressed. These properties have been measured by the adhesion tension of water for a film deposited on glass slides over a wide range of pressures. These results are shown in Fig. 4. The linear portion of the pressure-area curve of spread proteins from about 5 to 25 dynes has been described by Philippi (7) as a dehydration of the strongly bound water from the film; as the film is compressed, water is forced out. Over this range of pressures the adhesion tension steadily decreases, which is in accord with the idea that dehydration of the film is taking place. At the lower pressures, before this dehydration is experienced, there occur, however, some very interesting changes. The film goes from quite a hydrophilic state at a pressure of 0.5 dyne to a distinctly more hydrophobic one at 1.5 dynes. It seems reasonable to assume that both the hydrophilic and hydrophobic amino acid residues would tend, at low pressures, to lie horizontally on the surface, thus giving the peptide linkages the opportunity of coming into contact with water. As the pressure is increased from 0.5 to 1.5 dynes, the hydrophobic residues are forced out of the surface into the air, thus imparting a more hydrophobic character to the surface of the film. As the packing is continued up to 5 dynes, the film becomes more rigid and certain hydrophilic

residues are likewise forced out of the water, probably owing to valence angle requirements as the peptide chain is rotated from a horizontal to a vertical position. This appearance of hydrophilic groups in the upper surface of the film produces an increase in the hydrophilic character of the film, as is shown by changes in the adhesion tension. This assumption of orientation of the amino acid residues at low pressures is not in accord with explanation given by Philippi, who considers this portion of the curve to be characterized by a dehydration of the loosely bound water without orientation of the amino acid residues. Philippi (7) considers that surface potential measurements support his view. They would also support the view that orientation of the residues takes place at the low pressures; no doubt there is also some dehydration. Mitchell's (15) results with spread films of insulin, zein, and gliadin confirm the idea that orientation of the side chains takes place at low pressures.

It is questionable whether the smaller areas found at pressures above 25 dynes have any meaning as far as the structure of the film is concerned. Philippi (7) considers that protein is being forced out of the film above these pressures. If this were all that was happening, it would be expected that the film pressure would increase only slightly as the film area was diminished. Actually, however, as is shown in Fig. 1, at about 30 dynes pressure there is a considerable upswing of the pressure exerted by the spread native film (increasing pressure curve). It is well known that these highly compressed films are very viscous, and it is difficult to be certain that equilibrium film pressures are being measured; however, extended time was allowed for the film to come to equilibrium, and subsequent experiments (repeated four times) testified to the reality of the upward swing of the pressure-area curve. The adhesion tension studies also indicate certain structural changes in the film at these high pressures. It is clearly evident, if the reality of the higher pressure measurements be granted, that the area per amino acid residue is much too small for the peptide chain to lie flat on the surface with the amino acid residues oriented vertically both below and above the plane of the surface, however tightly packed they may be, since the area per molecule is much less than the sum of the average cross-sectioned areas of the residues, even though the residues are arranged alternately up

and down along the peptide chain. There must be a folding or crumpling of the entire peptide chain or of the film as the pressure is increased above 25 dynes. The hydrophilic properties of the film remain more or less constant at from 25 to 31 dynes. Beyond 31 dynes the film suddenly becomes more hydrophobic and continues to become progressively more so up to the rupture point.

Upon decreasing the pressure on this highly compressed film, considerable hysteresis is found. The compressibility, however, of this decreasing pressure curve (Fig. 1) is 0.0161 cm. per dyne, while that of the increasing pressure curve (Fig. 1) is 0.0164 cm. per dyne. This suggests that the two films have the same structure and that Philippi's explanation that protein is forced out of the film at higher pressures is at least partly right. In fact, by comparing the areas of the two films found by extrapolating the linear portions of the curves to zero pressure, it is found that about 30 per cent of the material in the film has been lost; the upward swing of the pressure-area curve at higher pressures is still unaccounted for, and it may be that the protein remaining in the film is folded up. The compressibilities of the spread native film undergoing compression were calculated over the entire range of pressures. Such calculation shows a minimum compressibility at 14 dynes with a sharp increase starting at 18 dynes and continuing to 29 dynes, at which point there again occurs a considerable decrease in compressibility which continues up to the rupture point. This again indicates structural changes in the films at high pressures.

As noted in the section on results, the compressibilities of both the native and heat-denatured proteins in compressed spread films are, within the experimental error, the same and also they are independent of the extent of spreading. This is taken to mean that compressed spread films of native and heat-denatured proteins have identical structure, and this structure is independent of the spread area. This indicates that where lack of spreading for native or heat-denatured protein is observed, the real reason for lack of spreading is that part of the material has been lost in the underlying buffer solution. The compressibilities of the urea-denatured films showed a peculiar dependence on the spread area (Fig. 3); the film being in general more compressible the smaller the spread area. If the linear portion of the pressure-area curves

reflects a dehydration of the film, this increase of compressibility would mean a more easily dehydrated film at the smaller areas. Since at maximum spread areas the compressed, spread native, urea-denatured, and heat-denatured films are so nearly alike as far as their spread areas and compressibilities are concerned, these three maximum spread films are probably identical.<sup>3</sup> The increase of the compressibilities of the urea-denatured film with decreasing spread areas may mean that some urea remained in the incompletely spread film which, along with water, is forced out of the film as the pressure is increased, giving rise to a greater compressibility of the film.

#### SUMMARY

1. A technique has been described for spreading urea-denatured and heat-denatured egg albumin in monolayers on a N/150 sodium acetate buffer at pH 4.9. The important point is to spread the denatured protein from a solution having the proper acidity.

2. Films of native, urea-denatured, and heat-denatured egg albumin have been studied with a Langmuir surface balance in respect to their compressibility and spread area. Under optimum conditions for spreading the properties of the urea- and heat-denatured films approximate very closely those of the spread native film, and the suggestion is put forward that surface-denatured (spread protein), urea-denatured, and heat-denatured proteins are structurally very similar and represent an unfolding of the native protein molecule in the form of an asymmetric polar molecule. Evidence is presented for believing that both urea- and heat-denatured proteins in clear aqueous solution are also very asymmetrical and are unfolded forms of the native molecule.

3. The compressibilities of the compressed spread native and heat-denatured protein films are found to be, within experimental error, the same. The compressibilities of monolayers of these spread native and heat-denatured proteins are independent of extent of spreading and of the pH of the solution being spread.

<sup>3</sup> The compressibilities, as calculated in this paper, apply to the compressibilities of the contracted films at zero pressure. As can be seen from Fig. 1, there is some difference between the extended films of spread native protein, heat-denatured protein, and urea-denatured protein but at higher pressures these three films compress to a very similar structure.

The compressibilities of film of urca-denatured protein are found to be a function of their spread area; in general, the poorer the spreading, the greater is the compressibility.

4. The hydrophilic properties of the air side of films of spread native protein have been investigated by means of contact angle studies on the protein films deposited at various film pressures on glass slides. The changes of the hydrophilic character of the film with increasing pressures have been related to the orientation of the amino acid residues and to a dehydration of the film.

5. It has been found possible to surface-coagulate heat-denatured egg albumin by shaking a clear solution of heat-denatured protein.

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## THE COMPOSITION OF ELASTIN\*

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Elastin is one of the prominent fiber proteins in the body, yet one of the least well known. Very little work has been done upon its chemical and physical characterization since the early work of Richards and Gies (1) in their study of ligament, of Abderhalden and Schittenhelm (2), who accounted for 61 per cent by weight of the amino acids in elastin, and of Kossel and Kutscher (3), who studied the basic amino acid content. It seemed probable that application of some of the more recently developed methods in protein chemistry might yield interesting information.

### EXPERIMENTAL

Elastin was prepared from the ligamentum nuchæ of the ox, by the method of Richards and Gies (1) modified in certain details. The use of saturated  $\text{Ca}(\text{OH})_2$ , recommended by them, was avoided, to obviate the possible destructive action of this reagent. The ligaments were obtained fresh from the slaughter-house, freed as completely as possible from adhering tissue, and passed through a meat grinder in successive stages from the coarsest to the finest knife mesh. The resulting material was reduced to a powder by mixing with solid carbon dioxide and passing through a large machine feed grinder, whereby it remained frozen throughout the grinding process.

This crude material (400 gm., dry weight) was successively extracted in the cold with 5 per cent  $\text{NaCl}$  solution, cold water, and  $\text{m}/15$  phosphate buffer, pH 8.0. The extractions were made

\* This report is from a dissertation submitted by William H. Stein in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.



at temperatures below 10° to avoid denaturation of unextracted proteins which would thereby be rendered difficult to remove at subsequent stages of the procedure. These extractions were repeated in each case until the supernatant liquid gave a negative biuret test for two successive extractions. The removal of mucoid by the buffer was followed, as well, by the Molisch test. After all soluble protein material had been removed from the elastin by the above solvents, the collagen was removed by continuous extraction with boiling water, with frequent changes of solvent, until the extracting fluid gave no biuret reaction after two successive extractions. This last procedure frequently required 10 to 14 days.

To remove fats ten to eleven extractions with 1 liter portions of boiling alcohol were made; in the final extractions the amount of residue (20 to 40 mg.) obtained on evaporation no longer showed a tendency to decrease progressively. The material was then washed twice with acetone and dried in a vacuum desiccator. From 400 gm. of ligament 153 gm. of elastin were obtained, a yield of about 38 per cent.

On analysis Sample 1 gave C 52.4, H 7.2, N 17.1, S 0.17, and ash 0.22. Another sample (No. 2) of elastin prepared in the same manner gave C 52.6, H 7.1, S 0.15, and ash 0.25.

As a further check upon the method, a small sample of elastin was prepared by a different procedure. Trial experiments with the above samples indicated that elastin is entirely insoluble in boiling 40 per cent urea, while the other proteins of the tissue appeared to be soluble (4). Powdered ligament was boiled, with frequent changes of solvent, with about 50 times its weight of 40 per cent urea solution for 4 to 6 days. The extractions were repeated until the extract gave no Molisch test, and no precipitate with phosphotungstic acid. The sample was defatted with boiling alcohol, washed with acetone as before, and dried.

On analysis Sample 3 gave C 52.2, H 7.1, S 0.16, and ash 0.20.

The agreement of the analytical figures for the three preparations, coupled with additional physical evidence to be presented later, made it appear likely that a protein of reasonably constant composition and properties could be prepared by these methods. Sample 1 was used for all work on elastin unless otherwise specified.

Microscopically the elastin appeared homogeneous. It still

retained its fibrous character. The fibers, when wet, were elastic; on stretching they acquired marked birefringence under a polarizing microscope.

### *Characterization*

*Color Tests*—The following results were obtained with the various color tests: the Molisch, negative after 4 hours; xanthoproteic, faintly positive; Millon, negative; Hopkins-Cole, negative; alkaline plumbite, no blackening; nitroprusside (with and without cyanide), negative.

*Solubility*—The solubility of elastin in water, *m*/300 sodium acetate, and *m*/300 acetic acid, of pH approximately 5, 7, and 3.6 respectively, was determined by rotating bottles containing 200 cc. of the solutions and 25 mg. of protein samples in a thermostat at 25° for 40 hours. The solubility ranged from 0.03 mg. of nitrogen in the acetic acid to 0.01 mg. in water, corresponding to 0.9 and 0.3 mg. of protein per liter. The solubility of elastin was also qualitatively tested in the following solvents: 20 per cent HCl, 25 per cent H<sub>2</sub>SO<sub>4</sub>, 20 per cent NaOH (cold), *N* HCl, *N* H<sub>2</sub>SO<sub>4</sub>, *N* NaOH, formic acid, acetic acid (glacial and dilute), 90 per cent phenol, lithium bromide (concentrations 7 gm. and 1 gm. per 10 cc. of water) up to 2 weeks boiling, zinc chloride, Schweitzer's reagent. With the exception of the strong acids and alkalies, the solubilities were determined at boiling temperatures as well as in the cold. In all cases the biuret test was negative in the extracts, and there were no indications of dispersion or solution.

In view of the strikingly low solubility of the preparation in all these liquids it seemed highly improbable that any contaminating protein material could be present in our sample. Such material if present in appreciable amounts should be extracted by such solvents as boiling saturated lithium bromide, formic acid,<sup>1</sup> or 90 per cent phenol.

In order to test further the homogeneity of our preparations quantitative recovery experiments from a number of reagents at room temperature were performed (see Table I).

<sup>1</sup> Although elastin appears to be insoluble in cold 90 per cent formic acid, it is completely dissolved by this reagent in less than 24 hours at 95–100°.

As a criterion of identity and homogeneity of solid substances insoluble in most solvents, the refractive index should prove useful. The refractive index of four elastin preparations (three of ours and one prepared by Richards and Gies (1)) and of samples of the elastin recovered from the treatments recorded in Table I was determined by the Becke line method. The value in all cases was  $1.532 \pm 0.002$ . That of phenol-treated elastin is higher than 1.6.

The isoelectric point was determined on a finely powdered material by cataphoresis, in a cell of the Kunitz-Northrop type as modified by Abramson (6). M/300 acetate buffers were used,

TABLE I  
*Recovery in Solubility Experiments*

	Reagent	Sample	Recovered	Time
	cc.	mg.	mg.	hrs.
Formic acid mixture (5) (50	25	51	51	19
cc. formic acid, 98%; 12 cc.		56	56	41
absolute alcohol; 1 cc. alco-		55	53	89
holic HCl, 25%)		53	51	161
5% H <sub>2</sub> SO <sub>4</sub>	100	100	100	25
5% NaOH	100	101	96	24

and the pH was determined with the glass electrode. The isoelectric point was found to lie at  $\text{pH } 4.18 \pm 0.03$ .

Elastin is slowly digestible by the enzymes pepsin and trypsin.

#### *Analysis of Hydrolysates*

**Amino Nitrogen**—The ratio amino N to total N was found to reach a constant maximum after 16 hours hydrolysis with boiling 20 per cent HCl. The amino N was determined with KI in the acetic acid according to the modification of Kendrick and Hanke (7). 2 cc. of a hydrolysate containing 0.32 mg. per cc. of total nitrogen<sup>2</sup> were taken for analysis. The amino N was 0.268 mg. per cc., making the ratio amino N to total N = 0.84.

**Amide Nitrogen**—10 cc. of a hydrolysate, corresponding to 9.36 mg. of protein, were made alkaline with saturated Na<sub>2</sub>CO<sub>3</sub>,

<sup>2</sup> All nitrogen determinations, unless otherwise specified, were made by the micro-Kjeldahl method.

and the ammonia was aspirated into N/70 acid. 0.38 mg. of N was found, making the amide nitrogen 0.04 per cent by weight of the elastin used (0.23 per cent of total N).

*Basic Amino Acids*—An attempt was made to determine arginine, histidine, and lysine, by the method of Block (8). From 2.36 gm. of elastin, arginine was isolated as the flavianate (S 6.7, calculated 6.6). The yield, 46.4 mg., corresponds to 16.5 mg. of arginine. After correction for aliquots removed, and for the solubility of arginine silver, the figure for arginine becomes 1.0 per cent.<sup>3</sup>

It was not found possible to isolate any histidine. The solution after decomposing the silver precipitate gave a very faint Pauly test. Control experiments indicated that the amount of histidine necessary to give approximately the same color would amount to less than 0.05 per cent by weight of the sample taken. Inasmuch as the solution gave a faint blue color with the Folin phenol reagent, it is possible that slight traces of tyrosine were present. In any case, we are forced to conclude that histidine is not present in measurable quantities.

It was not found possible to isolate pure lysine picrate from the phosphotungstate precipitate. The picrate obtained melted with decomposition over a range of 185–195°. Lysine picrate melts at 262°. Subsequent experiments indicated that elastin contains large amounts of proline, which would be expected to contaminate both the phosphotungstate and picrate precipitates. Recrystallization of the phosphotungstate was of no avail.

*Proline*—10.0 gm. of elastin (dry weight) were hydrolyzed by boiling for 16 hours with 100 cc. of 1:1 HCl. The excess HCl was removed *in vacuo*. The solution was filtered and the proline was determined according to the method of Bergmann (10). The amount of proline rhodanilate obtained was 7.866 gm. and had the correct composition.

*Analysis*— $(C_{18}H_{14}N_6S_4Cr)(C_6H_{10}O_2N) \cdot H_2O$

Calculated. C 41.7, H 4.3, N 16.2

Found. " 41.4, " 4.2, " 16.2

This corresponds to 1.49 gm. of proline, or 14.9 per cent proline by weight. A 5 per cent correction for the solubility of proline

<sup>3</sup> A determination of arginine, kindly made by Dr. Samuel Graff by his micromethod (9) with arginase, yielded 0.93 per cent.

rhodanilate (as indicated by Bergmann) brings this figure up to 15.6 per cent. A duplicate determination gave 14.7 per cent, average 15.2 per cent.

*Glycine*—Glycine was determined by the revised method of Bergmann (11, 12). To the filtrate from the proline determination 0.5 cc. of pyridine was added. The crystalline precipitate of pyridine rhodanilate was filtered off, washed with water, and discarded. 5 to 10 cc. of concentrated HCl were added to the filtrate and washings, and the solution concentrated to a syrup *in vacuo* to remove the excess HCl. The solution was filtered and the volume adjusted to about 100 cc. 25 gm. of potassium trioxalatochromiate were added, 2 cc. of concentrated HCl, and 210 cc. of 95 per cent ethyl alcohol, with shaking. The mixture was shaken for 6 hours on a shaking machine, left in the refrigerator overnight, and filtered. The precipitate was washed with 80 cc. of a mixture of 3 parts of alcohol and 1 part of 0.1 N HCl, and sucked on the filter for 2 or 3 hours. It was then dissolved in water and the volume made up to 1 liter. 2 cc. of this solution were used for amino nitrogen determination (7). The solution contained 500 mg. of amino N, which is equivalent to 2.67 gm. of glycine. Experiments on pure glycine solutions indicated that the recovery is only 90 per cent. When this correction factor is applied, therefore, the figure is raised to 29.7 per cent. A duplicate analysis gave 29.0 per cent, average 29.4 per cent.

Glycine determinations were attempted by the methods of Rapoport (13) and Town (14). The former method in our hands proved unworkable. Rapoport reports 98 per cent yields for the oxidation by alkaline permanganate of glycolic acid, and using his method finds 31.6 per cent of glycine in elastin. One source of error in Rapoport's method lies in the oxidation of glycolic to oxalic acid, which, as shown by Evans and Adkins (15) and confirmed by us, leads to the production of much carbon dioxide. In four determinations of analytically pure glycolic acid by alkaline permanganate oxidation, according to Rapoport's directions, we could recover only 60 to 73 per cent of it as calcium oxalate. No difference in yield with varying lengths of time of oxidation was observed.

In our hands the method of Town (14) consistently gave 98 per cent yields from solutions of pure glycine, and from mixtures of

glycine and alanine. We find that in pure solutions nitranilic acid forms a sparingly soluble compound with histidine and that this salt is composed of equimolar quantities of nitranilic acid and histidine.

To 75 mg. of histidine monohydrochloride were added 0.5 cc. of 20 per cent HCl, 25 cc. of absolute alcohol, and 150 mg. of nitranilic acid in 5 cc. of alcohol. A precipitate formed immediately, which, after 24 hours in the refrigerator, was filtered off. It weighed 123 mg., corresponding to 49 mg. of histidine, or 82 per cent.

*Analysis*<sup>4</sup>— $(C_6H_7O_2N_2) \cdot C_6(OH)_2(NO_2)_2O_2$ . Calculated. C 37.4, H 2.85  
Found. " 37.3, " 2.93

Under the conditions outlined above the reagent forms with lysine a fine amorphous precipitate which passes through the filter. No precipitate is formed with *dl*-alanine, *l*-leucine, *l*-aspartic acid, *l*-glutamic acid, *dl*-serine, *l*-proline, *l*-hydroxyproline, *dl*-phenylalanine, and *l*-arginine.

In order to ascertain the extent to which the reagent coprecipitates histidine and lysine with glycine, a mixture having approximately the same composition as gelatin (10, 16) was made (8.7 per cent *dl*-alanine, 7.1 per cent *l*-leucine, 3.4 per cent *l*-aspartic acid, 5.8 per cent *l*-glutamic acid, 0.4 per cent *dl*-serine, 19.7 per cent *l*-proline, 14.4 per cent *l*-hydroxyproline, 1.4 per cent *dl*-phenylalanine, 9.1 per cent *l*-arginine, 0.9 per cent *l*-histidine, and 5.9 per cent *l*-lysine). A solution of 2.138 gm. of this mixture, with 3.3 cc. of concentrated HCl, was diluted to a volume of 25 cc. with water.

To 79.4 mg. of glycine were added 2 cc. of the above solution (equivalent to 171 mg. of amino acids), 2 cc. of water, 30 cc. of absolute alcohol, and 300 mg. of nitranilic acid in 5 cc. of absolute alcohol. A fine precipitate formed at once. No immediate precipitation occurred when 2 cc. of the same solution, without glycine, were similarly treated. Both solutions were placed in the refrigerator overnight, at the end of which time each flask contained a precipitate. That from the mixture containing glycine weighed 234 mg. (103 per cent of the theoretical amount cal-

<sup>4</sup> It was essential to mix the sample with copper oxide for combustion to avoid explosion.

culated for complete precipitation of the glycine, histidine, and lysine, and 116 per cent of that theoretically obtainable from the glycine alone). The precipitate from the mixture containing no glycine weighed 16.6 mg. (62 per cent of the calculated amount for complete precipitation of lysine and histidine).

*Hydroxyproline*—9.90 gm. of elastin were hydrolyzed and freed from proline as described above. In the filtrate from the proline rhodanilate precipitation hydroxyproline was determined according to the method of Bergmann (10). The yield was 140 mg., equivalent to 1.4 per cent hydroxyproline. The somewhat colored material contained 10.8 per cent N (calculated, 10.5 per cent N). Bergmann indicates that there are large losses involved in the isolation of hydroxyproline. In the case of elastin, the per cent of hydroxyproline is so low that it is difficult to estimate the magnitude of the loss. 1.4 per cent is taken as a minimal value.

*Alanine*—Abderhalden (2) reported 6 to 7 per cent alanine in elastin. Fischer and Abderhalden (17) isolated alanine peptides from partial hydrolysates. The elastin used in the first case was prepared by the method of Zollikofer (18), which is unsatisfactory; in the second case the method of preparation is not reported. In the present work an attempt was made to determine it by the method of Bergmann and Niemann (12). The method depends on precipitation with sodium dioxypyridate, which forms very sparingly soluble compounds with alanine, glycine, and proline. It was possible, therefore, to use the elastin hydrolysate after removal of proline and glycine by the ammonium rhodanilate and potassium trioxalatochromiate reagents. The results gave no indication of the presence of alanine. We are informed, however (Bergmann, private communication), that failure to obtain any alanine with the reagent cannot be regarded as conclusive evidence for its absence. We therefore wish to leave open the question of the presence of alanine.

*Valine*—25.4 gm. of elastin containing 4.343 gm. of nitrogen were hydrolyzed for 16 hours with 250 cc. of 20 per cent HCl. The excess HCl was removed *in vacuo*, and the remaining chloride ion removed with silver carbonate. The silver was removed with  $H_2S$ , and the filtrate concentrated to remove  $H_2S$ . A loss of 3 per cent of the nitrogen was involved in the above procedures.

The dicarboxylic acids were removed by the method of Jones

and Moeller (19). The treatment of the dicarboxylic acid fraction, which contained 50 mg. of nitrogen, will be described later.

The main fraction, after being freed from barium with sulfuric acid, contained 3.965 gm. of nitrogen. A partial separation of the amino acids was effected by the method of Brazier (20). The solution was boiled with  $\text{CuCO}_3$  in excess for half an hour, filtered hot, and the excess  $\text{CuCO}_3$  thoroughly washed with hot water. The filtrate and washings were concentrated *in vacuo*, and then on the steam bath to dryness. The sticky copper salts were granulated by grinding with acetone, filtered, dried *in vacuo* over  $\text{CaCl}_2$  overnight, and in the oven at  $110^\circ$  for 2 hours.

The thoroughly dried copper salts were stirred mechanically with three 250 cc. portions of water for 45 minutes each, the mixture being centrifuged after each extraction. The combined water extracts were concentrated to dryness and granulated as before. On reextraction with water, a small amount of insoluble material was removed and added to the fraction of insoluble copper salts. The water extract contained 3.15 gm. of nitrogen, leaving 850 mg. of nitrogen in the insoluble fraction.

The water-soluble fraction was freed of copper with  $\text{H}_2\text{S}$  in  $\text{HCl}$  solution and the pH adjusted to 2.5 to 3.0 with  $\text{HCl}$ . 750 mg. of flavianic acid were added, and the bulk of the arginine removed as flavianate in the usual way. Proline was next removed with ammonium rhodanilate, as already described. The yield was 17.00 gm. of proline rhodanilate, equivalent to 3.23 gm. of proline, or 13.4 per cent with the 5 per cent correction factor. Previous determinations directly on a hydrolysate had yielded 15.1 per cent corrected. Hence the losses up to this point in this fraction amount to 11 per cent.

After removal of excess reagent in the usual manner the chloride ion was removed with  $\text{Ag}_2\text{CO}_3$  and the solution concentrated *in vacuo* to remove the ammonia and pyridine. The solution was acidified strongly with  $\text{HCl}$ , concentrated to a syrup to remove the excess, and 3 cc. of concentrated  $\text{HCl}$  added to a volume of 300 cc. 100 cc. of 20 per cent phosphotungstic acid in 0.1 N  $\text{HCl}$  were added to the solution in the cold. The solution was then heated to boiling and allowed to cool slowly. The supernatant fluid gave no further precipitate with phosphotungstic acid. The phosphotungstate precipitate was allowed to crystallize in the refrig-



erator overnight, and then filtered and washed with 5 per cent phosphotungstic acid in 0.1 N HCl. The treatment of this fraction, the "lysine fraction," will be described later.

The filtrate from the above precipitation was freed from phosphotungstic acid by shaking out with amyl alcohol-ether in the usual way, and evaporated to a syrup to remove excess HCl. Glycine was removed in the manner already described. The glycine precipitate contained 1160 mg. of amino N, which corresponds to 6.20 gm. of glycine, or 24.4 per cent. On addition of the usual 10 per cent correction factor, the figure is raised to 27 per cent. This is an 85 per cent recovery based on the figure of 29.4 per cent obtained originally.

The dark colored solution now contained, in addition to amino acids, much potassium chloride derived from the potassium trioxalatochromiate, and small amounts of both the proline and glycine reagents. These were decomposed with excess of baryta, and the precipitated  $\text{Cr}(\text{OH})_3$  and  $\text{BaC}_2\text{O}_4$  removed. The barium was removed with  $\text{H}_2\text{SO}_4$ . The amino acid hydrochlorides were taken up with alcohol, the KCl filtered off, and the chloride removed from aqueous solution with  $\text{Ag}_2\text{CO}_3$ .

The water-clear, faintly acid solution was concentrated to a small volume *in vacuo*, and hot alcohol added. Crystalline material separated out and, after 24 hours in the refrigerator, was filtered off. The crystals were shiny plates which weighed 2.8 gm. and melted at  $297^\circ$  with decomposition (uncorrected). Valine has a melting point of  $315^\circ$  with decomposition (corrected).

*Analysis*— $\text{C}_5\text{H}_{11}\text{O}_2\text{N}$ . Calculated. C 51.2, H 9.5, N 12.0  
Found. " 50.3, " 9.6, " 12.9, ash 0.5

The material had an  $[\alpha]_D = +3.25^\circ$  in water; for valine  $[\alpha]_D = +6.25^\circ$ . The nitrogen value did not change on recrystallization. The phenylhydantoin crystallized in long needles from ether-petroleum ether; melting point  $128\text{--}130^\circ$  (corrected), with preliminary sintering. The melting point of active valine is  $131\text{--}133^\circ$  (corrected)(21), while that of inactive valine is  $124^\circ$  (22). The substance contained 12.6 per cent N; calculated for  $\text{C}_{12}\text{H}_{14}\text{O}_2\text{N}_2$ , 12.9 per cent N. The substance was dissolved in N NaOH and allowed to remain for 48 hours at room temperature in order to racemize it. On acidifying and recrystallizing from

ether-petroleum ether, a substance was obtained which melted at 163–164° (corrected). Phenylureido-*dl*-valine melts at 163.5° (corrected). This substance contained 11.9 per cent N; calculated for  $C_{12}H_{14}O_2N_2$ , 11.9 per cent N. On boiling for 5 to 10 minutes with 20 per cent HCl, the *dl*-valine phenylhydantoin was obtained, m.p. 124°. Evidently racemization was followed, in this case, by ring opening to yield the hydantoic acid.

The product thus appears to be a partially racemized valine. The amount of valine, corrected only for known aliquots removed, was 11.5 per cent. If an additional correction factor, derived from the known losses involved up to the glycine precipitation (which was only 85 per cent of the previously determined amount) be added, the percentage is raised to 13.5.

The filtrate from the valine precipitation contained about 0.3 gm. of N. The amino acids were isolated by reprecipitating with alcohol from neutral solution. The products proved to be mixtures, having similar properties.

The analysis of the precipitates gave C 42.5, H 8.2, N 13.6, m.p. 228–242°; the substance decomposed with effervescence.

It was not found possible to resolve the mixture by fractional crystallization of the free acids or their phenylhydantoins. The low melting point with effervescence recalls serine. An attempt was made to establish the presence of serine by converting it to pyruvic acid phenylhydrazone under the conditions of Bergmann and Delis (23), but none could be isolated. As in the case of alanine the question of the presence of serine must therefore be left open.

*Aspartic and Glutamic Acids*—The alcohol-insoluble barium salts, the precipitation of which was described earlier, were taken up in water and freed of barium with  $H_2SO_4$ . To the hot solution (25 cc.) containing 50 mg. of nitrogen, 300 mg. of cupric acetate were added. No crystals of copper aspartate separated even after the solution was concentrated to 10 cc. and left in the refrigerator for 5 days. Elastin, therefore, appears to contain no aspartic acid.

Accidental loss of material prevented further attempts to isolate glutamic acid. Abderhalden and Schittenhelm (2) reported 1.74 per cent pyrrolidonecarboxylic acid and 0.76 per cent glutamic acid. In terms of glutamic acid this would be about 2.7 per cent

glutamic acid, a figure which is compatible with the nitrogen in our fractions.

*Lysine*—The phosphotungstate precipitate was suspended in water, acidified, and extracted with amyl alcohol and ether. The solution contained only 37.5 mg. of nitrogen. An attempt was made to precipitate lysine as the monohydrochloride from alcohol with the aid of aniline. No precipitate could be obtained; nor was it possible to obtain any picrate. Lysine, if present at all, can comprise only a fraction of 1 per cent of the elastin molecule.

*Leucine Fraction*—This fraction, containing 850 mg. of nitrogen, is represented by the water-insoluble copper salts. Preliminary experiments indicated that the fraction was a mixture of at least two amino acids, probably more. Abderhalden and Schittenhelm (2) reported and Engeland and Biehler (24) confirmed the presence of leucine and norvaline. Kapeller-Adler (25), using her colorimetric method, has reported the existence of 3.34 per cent of phenylalanine. All of these amino acids form insoluble copper salts, and should, therefore, be present in the leucine fraction. Several attempts to resolve the mixture were without success. We were able to effect a partial separation of what appeared to be leucine and norvaline by means of the zinc salts. After the copper salts were freed of copper and inorganic ions, the solution was boiled for half an hour with freshly precipitated  $\text{Zn}(\text{OH})_2$ . The mixture was evaporated to dryness on the steam bath, granulated to a powder with acetone, dried *in vacuo* and in the oven at  $110^\circ$ , and extracted with water. After removal of inorganic ions from the water solution of zinc salts, it was possible, with alcohol as a precipitant, to obtain a 3 per cent yield of semicrystalline material, which contained 11.82 per cent of nitrogen (calculated for norvaline, 11.96 per cent). From the water-insoluble zinc salts a fraction, about 2 per cent of the total protein, was obtained having 10.76 per cent nitrogen (calculated for leucine, 11.69 per cent). The products, however, were obviously mixtures. The phenylhydantoin of the "norvaline" melted at  $123\text{--}129^\circ$ . Active norvaline phenylhydantoin melts at  $112^\circ$  (26), inactive at  $102^\circ$  (27).

The yields by the zinc salt procedures were very poor. With the methods at present available, it appears impossible to approach a quantitative separation of a mixture such as this. We are unable, therefore, to add much to the existing knowledge

concerning the composition of this fraction, which accounts for about 30 per cent of the elastin.

*Tyrosine and Tryptophane*—The method of Lugg (28) as adapted by Brand<sup>5</sup> for the use of the photometer gave tyrosine 1.63 per cent and tryptophane 0.0 per cent.

*Sulfur Distribution* (See Table II)—The Folin photometric determinations were carried out as described by Kassell (29).

TABLE II  
*Distribution of Sulfur in Elastin\**

Elastin hydrolyzed	Methionine		Cystine		Sulfate S
	Volatile iodide method	Homocysteine titration	Baernstein method	Photometric Folin method	
mg.	per cent	per cent	per cent	per cent	per cent
735	0.38	0.48	0.20		0.019
720.5	0.38	0.30	0.265		0.026
957.4				0.22	
Average....	0.38	0.39	0.23		0.02

*Sulfur Recovery*

	per cent
Methionine S.....	0.08
Cystine S.....	0.06
Sulfate ".....	0.02
Total S.....	0.16
Pregl method.....	0.16

\* The authors wish to express their thanks for the aid given by Miss Beatrice Kassell and Dr. E. Brand in the determinations performed by their modification of the methods of Folin and Baernstein.

The determinations by the Baernstein method (30) were carried out according to the modification of Kassell and Brand (31).

DISCUSSION

The analytical results are summarized in Table III.

Bergmann has recently shown (10, 12, 32, 33) that the amino acids in certain proteins bear simple numerical relationships to

<sup>5</sup> Brand, E., private communication.

one another, and on the basis of these findings has formulated his "frequency theory."

There are present in elastin only five amino acids for the determination of which simple, accurate, reproducible methods are available—glycine, proline, arginine, cystine, and methionine. If the percentages of these amino acids are referred to a gm. molecular basis, it will be seen (Table IV, Columns 4 and 6) that they bear a simple numerical relationship to one another.

TABLE III  
*Amino Acids in Elastin*

Amino acid	Per cent by weight
Glycine.....	29.4
Alanine.....	None found
Valine.....	13.5
Aspartic acid.....	0.0
Arginine.....	1.0
Lysine.....	None found
Histidine.....	0.0
Cystine.....	0.23
Methionine.....	0.38
Tyrosine.....	1.6
Tryptophane.....	0.0
Proline.....	15.2
Hydroxyproline.....	2.0
"Leucine fraction".....	30.0
Phenylalanine*.....	3.34
Amide N.....	0.04
Total.....	85.0

\* From data of Kapeller-Adler (25).

The average molecular weight of the amino acids liberated on the complete hydrolysis of elastin was estimated to be about 104. In the estimation of the average molecular weight of all the amino acids, the percentages of the amino acids for which no good analytical methods are available (leucine, norvaline, alanine, and serine) were calculated from the per cent nitrogen in the impure material isolated from the various fractions. From this it follows that the average residue weight is about 86, and it can be calculated that 100 gm. of elastin would yield 1.16 gm. molecules of an

average amino acid on complete hydrolysis. On considering the values in Column 4, Table IV, one finds that the amino acids listed comprise  $1/3$ ,  $1/9$ ,  $1/192$ ,  $1/576$ , and  $1/576$  of all of the constituent amino acids.

In addition it may be pointed out that the value for valine, which is not listed in Table IV, comes reasonably close to making the ratio proline to valine 1:1. Actually it is 1.0:0.87, a satisfactory agreement considering the inevitable losses in the isolation method. Hence, when more accurate determinations of valine

TABLE IV  
*Ratio of Amino Acids in Elastin after Hydrolysis*

Amino acid	Mol. wt.	Weight		Gm. molecule per 100 gm. protein		No. of residues	Fraction of total residues
		Found	Calculated	Found	Calculated*	Ratio	Frequency
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
		<i>per cent</i>	<i>per cent</i>				
Glycine.....	75	29.4	29.4	0.392	0.392	192	3
Proline.....	115	15.2	15.0	0.132	0.131	64	9
Arginine.....	174	1.0	1.06	0.0057	0.0061	3	192
Cysteine†.....	121	0.23	0.24	0.0019	0.0020	1	576
Methionine.....	149	0.38	0.30	0.0026	0.0020	1	576

\* Base = 0.392 gm. molecule of glycine; 0.131 gm. molecule of proline.

† The cystine sulfur has been calculated as cysteine.

become possible, it may be found that it too comprises one-ninth of all the amino acids in elastin.

Examination of Columns 6 and 7 leads to the conclusion that elastin must contain 576 amino acid residues or some whole multiple thereof. When the number of units is multiplied by the average residue weight (86), it is found that elastin has a minimum molecular weight of about 49,500.

It may be pointed out that the ratios of amino acids in elastin fall into the series  $2^n \times 3^m$ , and the number of amino acid residues (576) is the same as Bergmann and Niemann (32) found for hemoglobin and fibrin, and twice 288, the number of residues found in egg albumin. Thus we have in elastin another protein which fits the "frequency theory" in so far as data are available.

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# THE MICRODETERMINATION OF THYROXINE IN THE THYROID GLAND OF THE NEW-BORN

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Elmer and Scheps (1) in 1935 determined the thyroxine and diiodotyrosine iodine in a series of thyroid glands from the human fetus and the new-born. The thyroxine iodine values ranged from 0.4 to 9.0 mg. per cent (dry gland) in ten cases, while the total iodine values varied from 1.3 to 20.5 mg. per cent (dry gland) in seven cases. The thyroxine constituted from 26 to 69 per cent of the total iodine. Of the eleven thyroxine iodine values given, nine were obtained by the method of Harington and Randall (2) and two by Elmer's (3) modification of the method of Leland and Foster (4). No total iodine values were given for the latter two, so the ratio of thyroxine iodine to total iodine could not be calculated.

For reasons given elsewhere (4) we believe that the thyroxine values obtained by the method of Harington and Randall include a part of the diiodotyrosine present in the gland and are higher than the true value. We have therefore redetermined the thyroxine iodine values of a series of new-born and fetal thyroids, employing an adaptation of the method of Leland and Foster.

## *Method*

It was necessary to resort to microtechnique for the determination both of thyroxine and of total iodine. The microadaptation of the method of Leland and Foster, devised by Foster (5), was used in obtaining the thyroxine values. Analyses, both for thyroxine I and total I, were carried out in duplicate. The thyroid gland material was dried initially at from 78–80° and brought to constant weight at 60°. Samples of from 25 to 50 mg. were hy-



drolyzed for 18 hours with 4 cc. of 2 N NaOH in a 2 N  $\text{H}_2\text{SO}_4$  bath. The tubes in which the hydrolysis was carried out were constricted near the bottom to form a bulb of 5 cc. capacity instead of 2 cc., as described by Foster, since our samples were considerably larger than his. An effort was made to keep the ratio of alkali to gland material as nearly like that in the macromethod as possible.

After cooling, sufficient 2 N NaOH was added to bring the level up to the constricted part of the tube. The thyroxine was then extracted from the alkali by being shaken twice with an equal volume of butyl alcohol. The butyl alcohol fractions were withdrawn, in turn, by means of suction through a microsiphon into a second similar tube with a bulb of 10 cc. capacity. The combined extracts were washed with an equal volume of 1 N NaOH and centrifuged gently. The butyl alcohol layer was siphoned into a 100 cc. digestion flask, while the NaOH was again extracted with half its volume of butyl alcohol. After the mixture was centrifuged, the alcoholic layer was combined with the original butyl alcohol fractions.

From this point on the method differs from that described by Foster. The butyl alcohol was evaporated off under reduced pressure and the iodine determined in the residue by the method of Trevorrow and Fashena (6). The alkaline residue was dissolved in 10 cc. of iodine-free water. 1 gm. of iodine-free potassium dichromate, 10 mg. of cerous sulfate, and 10 cc. of chromic acid-sulfuric acid were added. The sides of the flask were washed down with 15 cc. of redistilled water, a glass bead was added, and the mixture was heated to  $195^\circ$ . After cooling to  $70^\circ$ , 20 cc. of water were added and the mixture again heated to  $195^\circ$ . The flask was again cooled to  $70^\circ$  and the mixture transferred to a 500 cc. digestion flask with the aid of 125 cc. of redistilled water. 5 cc. of phosphorous acid (Baker and Adamson, reagent quality) were added when the mixture had cooled to  $50^\circ$  and the flask was immediately connected to the distillation apparatus described by Trevorrow and Fashena. We experienced some difficulty in obtaining quantitative recoveries by the use of this apparatus. Under the following conditions we were consistently successful in recovering known solutions of iodine in amounts up to 6 micrograms. We sometimes recovered 12 but never succeeded in recovering 25 micrograms (Table I). Because of this condition

we were careful in our series to repeat with smaller samples those determinations in which the iodine values were found to be above 6 micrograms.

A gentle current of alkali-washed air was drawn through the apparatus while the digestion mixture was heated to the boiling

TABLE I  
*Total Iodine Recoveries in Known Solutions of Potassium Iodide*

Total I present	Total I found	Recovered
<i>micrograms</i>	<i>micrograms</i>	<i>per cent</i>
1.24	1.22	98
1.24	1.30	105
2.54	2.66	105
2.54	2.52	99
2.54	2.66	105
2.54	2.63	104
2.54	2.63	104
2.54	2.54	100
2.54	2.57	101
2.54	2.49	98
2.54	2.63	104
2.54	2.49	98
6.35	6.07	96
6.35	6.29	99
6.35	5.99	94
12.70	12.30	97
12.70	10.30	81
12.70	10.60	83
12.70	12.20	96
12.70	11.80	93
25.40	17.50	69
25.40	19.50	77
25.40	19.60	77
25.40	19.60	77
25.40	21.90	86

point. The suction was then increased so that the air was drawn rapidly through the mixture. The flame was adjusted so that not less than 100 cc. (better 110) of distillate were collected in from 45 minutes to 1 hour. A small thermometer (range 120–180°) was suspended from a glass hook in the distillate. Care was taken that the temperature did not exceed 140° during the distillation.

The distillate was transferred to a 125 cc. Erlenmeyer flask and loosely stoppered by a glass bulb with an opening in its side. This prevented loss both from spattering and from drying on the side of the Erlenmeyer flask. Three glass beads were added and the liquid boiled down to 10 or 12 cc. in alkaline solution (5) over a steady flame. 1 drop of methyl orange was added, 3 per cent  $\text{H}_2\text{SO}_4$ , until acid, and bromine vapor. Boiling was continued very carefully over a low flame with the flask still covered until a volume of 4 or 5 cc. was reached. Quantitative transfer was then made by means of 6 cc. of redistilled water to a titration tube 12 cm.  $\times$  2.5 cm. Boiling was continued over a small microburner flame until a volume of 2 cc. was reached. The boiling tube was

TABLE II  
*Comparison between Thyroxine Iodine Values Found by Micro- and Macroanalysis of Burroughs Wellcome Thyroid*

Macroanalysis, calculated	Microanalysis, found	Recovered
<i>micrograms</i>	<i>micrograms</i>	<i>per cent</i>
3.05	2.98	98
4.23	3.98	94
2.36	2.43	103
2.40	2.52	105
3.74	3.50	94
2.23	2.27	102
2.51	2.45	98

kept covered by the same small glass bulb used on the flask. After cooling, 1 drop of phenol and 2 drops of a solution of KI containing 10 mg. per drop were added and the solution titrated with 0.001 N sodium thiosulfate until the yellow color had almost disappeared. 1 drop of a 1 per cent solution of potato starch saturated with sodium chloride was then added and the titration completed. A modified Rehberg microburette graduated to 0.001 cc. was used. With the titration tube described the very small volume of liquid was more readily seen and the disappearance of the blue color more accurately judged than is possible when the titration is carried out in the 125 cc. volumetric flask in which the first boiling is done (as described by Trevorrow and Fashena). In our opinion, also, the end-point, which at best is hard to determine satisfactorily, is sharper in the presence of 1 drop of 90 per cent phenol.

In Table II are recorded the thyroxine values obtained both by micro- and macroanalysis. For our "known substance" we used a commercial preparation of Burroughs Wellcome thyroid which had been repeatedly analyzed in two laboratories and found to contain by macroanalysis (4) 0.0592 per cent thyroxine iodine. It will be seen that the agreement between the two methods is satisfactory. Samples varying from 4 to 7 mg. were used for analysis.

### *Material*

A series of eighteen human thyroid glands collected from October to May inclusive was obtained for analysis. Two were from 7 month fetuses, fourteen from still-born infants, one from a 2 day-old and one from a 12 day-old infant. No data concerning the mothers were available and no further data concerning the infants, except the weight in the majority of cases. Histological examination was made of three representative glands (Cases 13, 14, and 16). The structure was undifferentiated, without definite follicle formation. Little or no colloid was present. Slight hyperplasia was observed in one gland (Case 16) but none in Cases 13 and 14.

### *Results*

The total iodine and thyroxine iodine values obtained for the series are collected in Table III. The values obtained from the analysis of the gland in Case 8 are exceptionally high. Although we have no data on this point, it is possible that the mother had received iodine either as a therapeutic agent or as iodized salt, which might account for the high results found. This gland has been excluded in calculating the averages. The fresh weights of the remaining seventeen thyroid glands range from 0.45 to 3.00 gm. with an average of 1.43 gm. The dry weights vary between 0.049 and 0.555 gm. with a mean value of 0.246 gm. The thyroxine iodine values for sixteen of the glands range from 14 to 209 micrograms per gm. of dry weight. In one gland no thyroxine iodine was found. The average for the seventeen glands was 61 micrograms. The mean content of total iodine is 254 micrograms, with values ranging from 35 to 654. The thyroxine per cent of total iodine was calculated to be 20.0.

establish the presence of thyroxine in the thyroid gland of the fetus and the new born infant. A direct comparison between our figures and those of Elmer and Scheps is difficult, since our series is made up mainly of term infants and theirs of fetuses from 3½ to 6 months with only three term infants included.

TABLE III  
*Thyroxine Iodine and Total Iodine in Thyroids of New-Born*

Case No.	Age	Weight /	Weight of gland		Total iodine		Thyroxine iodine		Thyroxine I Total I per cent
			Fresh	Dry	Per gm. dry weight	In whole gland	Per gm. dry weight	In whole gland	
		lbs.	gm.	gm.	micro-grams	micro-grams	micro-grams	micro-grams	
1	7 mo. fetus	2.80	0.52	0.0949	133	12.6	29	2.8	21.8
2	7 " "	4.50	0.64	0.1290	242	31.2	50	6.5	20.7
3	Still-birth	7.25	0.80	0.1617	599	96.9	149	24.1	24.9
4	"	10.00	2.20	0.4181	89	37.2	14	5.9	15.7
5	"	4.75	0.45	0.0494	259	12.8	50	2.5	19.3
6	"		2.85	0.5151	35	18.0	0	0.0	0.0
7	"	9.50	2.20	0.3952	152	60.1	30	11.9	19.7
8	"		1.20*	0.2303*	1680*	386.9*	469*	108.0*	27.9*
9	No data		0.70	0.1119	176	19.7	38	4.3	21.6
10	Still-birth		3.00	0.5550	177	98.2	33	18.3	18.6
11	"	7.50	0.76	0.1284	311	31.9	82	10.5	26.4
12	"	8.50	1.70	0.2819	163	39.9	32	9.0	19.6
13	"	7.00	1.50	0.2809	90	25.3	15	4.2	16.7
14	"	9.00	0.98	0.1594	220	35.1	33	5.3	15.0
15	No data		1.10	0.1962	654	128.3	209	41.0	32.0
16	Still-birth	7.50	1.10	0.1690	352	59.5	84	14.2	23.9
17	2 days	8.00	1.42	0.1360	524	71.3	168	22.8	32.1
18	12 "		2.40	0.4029	146	58.8	18	7.3	12.3
Average.....			1.43	0.2462	254	49.2	60.8	11.2	20.0

\* Excluded from the average.

For purposes of comparison we have assembled in Table IV the mean thyroxine and total iodine values of our series, those of Elmer and Scheps, and of Lelkes (who reports only total iodine), all expressed in terms of micrograms per gm. of fresh weight.

From Table IV it may be seen that our average total iodine

values are much higher than those found by Elmer and Scheps. For seventeen new-born and fetal thyroids we obtain an average of 41 micrograms per gm. of fresh gland, a value 3.5 times that found by Elmer and Scheps for their series, and 15 times their value for the new-born. In terms of micrograms present in the whole gland our value of 49.2 is more than 4 times as great as their value of 11.0 for the new-born. Geographical differences may account in part for our higher values but it seems improbable that all of the difference may be attributed to this factor. Possibly the higher figures may be due largely to the different

TABLE IV  
*Comparison of Values for Thyroxine and Total Iodine Found by  
Different Investigators*

	Mean total iodine		Mean thyroxine iodine	
	Per gm. fresh weight	In whole gland	Per gm. fresh weight	In whole gland
	micrograms	micrograms	micrograms	micrograms
Elmer and Scheps				
Whole series.....	12.4	6.3	4.4	3.1
New-born.....	2.8	11.0	1.4	6.8
Lelkes				
Whole series.....	20.6	22.9		
New-born.....	4.0	19.5		
Palmer, Leland, and Gutman				
Whole series.....	41.0	49.2	9.9	11.2

technique employed, that of oxidation by dichromate in acid solution followed by aeration of the iodine in a closed system.

Our thyroxine iodine values are also higher than those of Elmer and Scheps. In terms of micrograms per gm. of fresh weight our value of 9.9 is seen to be more than twice as high as their whole series and 7 times as high as their values on the new-born. In comparing the thyroxine iodine present in the whole gland we find nearly 4 times as much as for their total series and nearly twice as much as for their series on the new-born. This is interesting in view of the fact that values obtained by the Leland and Foster method of analysis tend to be lower than those by the Harington and Randall method.

We find the ratio of thyroxine iodine to total iodine to be 20.0 per cent, a figure not much lower than that found in a series of 52 normal adults, 25.2 per cent (5). In other words, the ratio in new born infants is essentially the same as in normal adults, although the absolute amount of thyroxine, per cent of dry weight of gland, is much less. Elmer and Scheps find 47.3 per cent of their total iodine present as thyroxine, a value more than twice as high as ours although within normal adult limits as determined by Harington and Randall.

The total iodine content of the fetal and new-born thyroid has been determined many times during the past 40 years. Some investigators, particularly in earlier years, report the absence of iodine in fetal glands. An adequate survey of the literature has been made by Lelkes (7) who made a study of fetal and infant thyroids in 1934. Thirty-four cases, ranging in age from a fetus of 2.5 months to an infant of 8 months, were analyzed for total iodine by Scheffer's method (8). As may be seen from Table IV, Lelkes finds an average of 20.6 micrograms per gm. of fresh thyroid gland, about half of the value found for our series. When our figures are compared only with those for the new-born included in his series, our average is 10 times as high as his. In terms of total iodine present in the whole gland we obtain a value about 2.5 times that for his new-born. The great difference in values noted may be attributed chiefly to the different methods employed. Results obtained in this laboratory by the use of methods involving ashing with alkali and extraction with alcohol were unsatisfactory. Recoveries were low, particularly of organic iodine compounds. Attempts to recover diiodotyrosine and iodized egg albumin were consistently unsuccessful even when the initial ashing temperature was carefully controlled by means of a muffle furnace.

#### DISCUSSION

Our data, supported by that of Elmer and Scheps, suggest but do not prove an early independent function of the thyroid gland in fetal life. Certainly it has been demonstrated that thyroxine exists in the thyroid gland as early as the 3rd month. Furthermore toward the end of fetal life the percentage of the total iodine present as thyroxine iodine is essentially that of adults. The actual amount of thyroxine in the gland is low. However,

the metabolic requirement of the fetus is small. Therefore it seems probable that during most of the fetal life, thyroxine, so necessary to normal development and as a metabolic stimulus, is derived from the thyroid gland of the fetus and not from the mother.

#### SUMMARY

1. Eighteen thyroid glands obtained from new born infants were analyzed for their total and thyroxine iodine contents by a microadaptation of the method of Leland and Foster.

2. The average total iodine content was 254 micrograms per gm. of dry weight with a maximum of 654 and a minimum of 35.

3. The average thyroxine iodine content was 61 micrograms per gm. of dry weight with a maximum of 209 and a minimum of 0.

4. The thyroxine per cent of total iodine was 20.0, substantially the same as the value 25.2 found in a series of 52 normal adult thyroids.

The authors wish to express to Dr. G. L. Foster their appreciation of his interest and assistance in carrying out the work.

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## THE VERATRINE ALKALOIDS

### IV. THE DEGRADATION OF CEVINE METHIODIDE

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Freund and Schwarz (1) in their careful investigation of the preliminary phases of the chemistry of cevine demonstrated its tertiary character by the formation of a methiodide  $C_{27}H_{43}O_8N \cdot CH_3I$ . Somewhat later Freund (2) confirmed this by the production of a crystalline oxide with hydrogen peroxide, and since this is a property not exhibited by pyridine or quinoline bases but by alkylated piperidine bases or dialkylamines he concluded that the nitrogen of cevine may belong to a double ring system such as was assumed in dihydroberberine. This suggestion has since received some support in the isolation by us (3), from the hydrogenated products of the soda lime distillation of cevine, of a base  $C_{10}H_{19}N$  which is either an octahydropyrrocoline or octahydropyridocoline derivative. On attempting to proceed further with the degradation of the methiodide by the usual procedure, Freund and Schwarz (4) were blocked by the unexpected behavior of the base obtained from it with alkali, the so called des-base  $C_{28}H_{45}O_8N$ . This formula was well established by analysis of a number of its salts. However, contrary to what should have been expected, it could not be hydrogenated and attempts to prepare a methiodide were also unsuccessful. The suggestion was offered that the so called des-base forms a pseudobase.

In connection with our own study of the degradation of cevine we have had occasion to repeat the preparation and study of the base from cevine methiodide. While we have duplicated essentially the data given by Freund and Schwarz, we have been forced to a different interpretation of its nature. The formula  $C_{28}H_{45}O_8N$  has been confirmed by the analysis of the base itself

which separates in anhydrous form from absolute alcohol. Also all attempts to demonstrate the presence of a double bond by hydrogenation were unsuccessful and no methiodide could be prepared from it. In contrast to cevine it is completely insoluble in ether. Cevine can be slowly distilled unchanged at very low pressures, whereas the des-base shows no tendency to distil. The des-base also as shown by Freund and Schwarz forms a bicarbonate, while cevine, according to our experience, does not form a stable carbonate. The physical and chemical properties of this substance suggest that it must be an internal salt or betaine.

Cevine itself has been shown by Freund to form a potassium compound,  $C_{27}H_{41}O_8NK_2$ , which is readily decomposed by carbon dioxide. Hess and Mohr (5) suggested a somewhat different formula for this derivative; *viz.*,  $C_{27}H_{42}O_8NK \cdot KOC_2H_5$ . The possibility that this potassium salt results from the cleavage of a lactone group in cevine, as suggested by Macbeth and Robinson (6), appears incompatible with the reported analytical results with the potassium derivative. An additional mole of water would be necessary in the make-up of such a potassium derivative. It is also incompatible with the formula derived for the des-base. The fact that the des-base separates as such and not as a potassium compound as in the case of cevine points to an internal neutralization of whatever the negative group is in the latter which forms such a potassium salt. An enolic (or phenolic) betaine appears to fit best into the general picture. Since the liberation with alkali of the quaternary hydroxide itself from cevine methiodide does not at once give the crystalline betaine, but only on heating, it is probable that some rearrangement is necessary before betaine formation can occur.

Evidence of the enolic betaine nature of this base has been obtained by the comparison of the dissociation constants of cevine itself and of the base in question. We are greatly indebted to Dr. Theodore Shedlovsky for measurements made, with the glass electrode, by determining in each case the pH of a solution of the base half neutralized with hydrochloric acid. The  $pK'_B$  of cevine proved to be 9.48, while in the case of the des-base the pH measurement showed it to be completely dissociated like an alkaline hydroxide. The nitrogen must therefore still be in quaternary form, since quaternary hydroxides are known to

have a strength comparable to an alkaline hydroxide. An enolic or phenolic betaine should exhibit in aqueous solution such properties because of the very feebly acidic character of an enolic or phenolic group.

It has not been possible to obtain conclusive evidence of the presence of a phenolic group in the cevine molecule. Similarly a carbonyl group was not directly detectable by the usual reagents. However, the possible presence of the latter has been indicated by hydrogenation experiments with Raney's nickel catalyst. Earlier attempts to hydrogenate cevine with platinum oxide catalyst had been unsuccessful. Raney's nickel catalyst in methyl alcoholic solution caused the absorption of 1 mole of hydrogen. This was confirmed by isolation of a crystalline reaction product which analysis showed to be  $C_{27}H_{45}O_8N$ . It was also found possible to reduce cevine with sodium and butyl alcohol to a crystalline substance,  $C_{27}H_{45}O_8N$  (or possibly  $C_{27}H_{47}O_8N$ ), which was not identical with the hydrogenation product. As a by-product a substance was also obtained in small amount, the analysis of which agreed with a formula  $C_{27}H_{45}O_7N$ . Further study of these substances is now in progress.

In our investigation of methods to accomplish further degradation of the betaine it was found necessary to employ more vigorous conditions, as in the case of cevine itself (3). Distillation with soda lime was found to give a mixture of bases and non-basic material. As with cevine, the basic fraction proved to be unsaturated and unstable and represented only a fragment of the alkaloid molecule. For its successful investigation it was necessary to stabilize it by hydrogenation. On fractionation the major portion of the resulting basic material was found to consist of an N-methyl hydroxy base,  $C_9H_{13}ON$ . This substance was not purified as such but after conversion into its methiodide,  $C_{10}H_{22}ONI$ , which had excellent properties. The latter proved to be optically active ( $[\alpha]_D^{20} = +14.5^\circ$  in alcohol) and now contained two N-methyl groups. The Zerewitinoff determination showed the presence of a hydroxyl group. The original N-methyl base must therefore be a piperidine (or pyrrolidine) derivative. It is in all probability a hydroxy derivative of N-methyl-2-ethyl-5-methylpiperidine corresponding to the methylethylpiperidine previously obtained from cevine with soda lime (3). Our attempt

to test this possibility by dehydrogenation with zinc dust was unsuccessful because of complications caused by the N-methyl and hydroxyl groups.

The attempt was made to carry this substance through additional steps of exhaustive methylation. The dimethyl quaternary hydroxide liberated with silver oxide was found to decompose readily at 80–90° and 15 mm. to give a volatile base which, contrary to expectations, could not be hydrogenated. Again the resulting base was not isolated as such but as the crystalline methiodide. This salt is a trimethyl-substituted ammonium iodide. Analytical results agreed with the formula  $C_{11}H_{24}ONI$ . Unlike its precursor, it gave only a fraction of a mole of methane with Grignard's reagent. Since the failure of attempts to hydrogenate this salt confirmed its apparent saturated character, it appears that as the quaternary hydroxide suffers ring cleavage the hydroxyl group which attaches itself to the carbon atom cleaved from the nitrogen atom is split off not to form the double bond of a des-base but to form an oxide ring with the hydroxyl group already present in the parent base. The methiodide must therefore be an open chain oxidotrimethyl-substituted ammonium iodide. Its nature and the fact that it is optically active make its exact identification by synthetic procedures difficult. A preliminary attempt at the final step in the exhaustive methylation has given trimethylamine and a fragrant volatile oil which was obtained in a yield too small for study.

No evidence was obtained of the formation from the betaine with soda lime of the dicyclic base,  $C_{10}H_{19}N$ , which we obtained from cevine (3). This cannot be regarded as evidence that such a dicyclic base is an artifact produced by secondary cyclization and does not occur in cevine. It would be expected that the N-methyl quaternary salt of such a dicyclic base would degrade preferably by ring cleavage and formation of an N-methyl tertiary base such as actually observed.

#### EXPERIMENTAL

*Cevine Methiodide*—For the preparation of this substance a slight modification of the method as given by Freund and Schwarz (4) was employed. The yields (98 per cent of the theory) reported by these workers could not be duplicated by us. Although

many modifications were tried of conditions under which cevine was allowed to react with methyl iodide, the highest yield of recrystallized methiodide isolated was roughly 75 gm. from 100 gm. of cevine. This was best obtained by allowing a solution of 100 gm. of cevine in 100 cc. of methyl alcohol and 70 cc. of methyl iodide to stand about 18 hours at 25°. After evaporation of the solvent and excess reagent, addition of water caused copious crystallization of the methiodide which was collected and then recrystallized from a minimal volume of water. It melted at 253–257° with slow gas evolution after preliminary softening above 245° (Freund and Schwarz reported 257° with decomposition).

*Cevine Methochloride*—This substance was prepared by shaking an aqueous solution of the methiodide with an excess of silver chloride. The solid obtained after evaporation of the filtrate was recrystallized from absolute alcohol. It formed platelets which melted at 280–283° and was anhydrous. It showed a very low rotation in aqueous solution.

$$[\alpha]_D^{25} = -1.5^\circ \text{ (} c = 2.0 \text{ in water)}$$

$C_{23}H_{45}O_3NCl$ . Calculated, C 60.02, H 8.28; found, C 60.02, H 8.56

*The Betaine  $C_{23}H_{45}O_3N$  (So Called Des-N-Methyl Cevine)*—This substance was prepared from the methiodide essentially as described by Freund and Schwarz (4) with the exception that it was found unnecessary to liberate the quaternary hydroxide with silver oxide before heating with KOH.

76 gm. of the methiodide were dissolved in a solution of 75 gm. of KOH in 225 cc. of  $H_2O$  and the mixture was heated on the water bath in a flask through which N was passed. After  $\frac{1}{2}$  hour the thick crystalline pap was cooled and the crystals were collected on a Jena funnel, washed with 25 per cent KOH, and at once dried in an exsiccator. Since the base is less soluble in hot alkali than when cool, the mother liquor was again heated. A second crop of crystals was obtained which was collected while hot. The first crop of crude base weighed 51 gm. and the second 14 gm.

For purification the base was suspended in a small volume of water and the colorless, lustrous leaflets were again collected. After it was sucked almost dry, the product was washed with absolute alcohol. Although all attempts by simple means to

recrystallize the base failed, the following device was found to effect recrystallization. A portion of the base was dissolved in hot, carefully diluted alcohol. The solution was then repeatedly boiled down to smaller volume after addition of absolute alcohol. The base separated as a crystalline powder which was collected with absolute alcohol. It melted with decomposition at 273–275°.

$C_{23}H_{45}O_2N$ . Calculated, C 64.20, H 8.67; found, C 63.78, H 8.66

Freund and Schwarz have described the free base as a hydrate,  $C_{23}H_{45}O_2N \cdot H_2O$ .

The hydrochloride of the base was obtained by solution in absolute alcoholic hydrochloric acid as lustrous rhombic platelets which melted with effervescence at 242°. It was found to contain 1 mole of alcohol which was not removed on drying at 100°.

$$[\alpha]_D^{25} = -28.5^\circ \quad (c = 1.99 \text{ in water})$$

$C_{23}H_{45}O_2NCl \cdot C_2H_5OH$ . Calculated. C 59.42, H 8.65,  $OC_2H_5$  7.42  
Found. " 59.80, " 8.87, " 7.03

This salt was similarly found to separate from methyl alcohol with the solvent of crystallization and melted with effervescence at 248–250°. The solvent was not removed at 140°.

$C_{23}H_{45}O_2NCl \cdot CH_3OH$ . Calculated. C 58.80, H 8.51,  $OCH_3$  5.24  
Found. " 59.27, " 8.42, " 4.79

*Soda Lime Distillation of the Betaine,  $C_{23}H_{45}O_2N$* —This operation was performed with the crude, well washed betaine after careful drying in an exsiccator. An intimate mixture of 15 gm. with 120 gm. of soda lime was heated in a glass distilling bulb immersed in a nitrate bath. A slow stream of  $H_2$  was passed through the apparatus during the operation. Following the initial distillation of water, signs of decomposition were evident at 240° but at 260° a copious distillation of almost colorless oil occurred. After several hours the temperature was raised to 310° when a dark brown, more viscous oil distilled. After 1½ hours at this temperature the operation was discontinued.

The distillate was transferred to a hydrogenation apparatus with alcohol and about 10 cc. of acetic acid and hydrogenated with 0.1 gm. of Adams and Shriner's platinum oxide catalyst.

The absorption of  $H_2$  was at first rapid but persisted on longer shaking, so the operation was continued for about 17 hours. The total absorption, exclusive of catalyst, varied in different experiments from 235 to 260 cc. The solution was acidified with an excess of HCl and the mixture was distilled with steam to remove volatile non-basic material. Following this, the residue was made alkaline and the steam-volatile bases were collected in dilute HCl. Very little resinous residue remained undistilled.

The solution of the bases on concentration gave a syrupy residue of HCl salts which colored red on standing. Two experiments corresponding to 30 gm. of starting material were combined at this point and the base was liberated with 35 per cent KOH.

TABLE I

B.p., 760 mm.	Weight	Analysis	
		C	H
°C.	gm.	per cent	per cent
145	0.17		
174	0.175		
200	0.185	72.59	12.30
208	0.175	70.07	11.81
208	0.175	69.24	11.92
209	0.185	68.87	11.80
209	0.185	69.22	11.75
208	0.185	70.60	12.28
211	0.185	71.05	12.07

About 3 cc. of a red-colored, basic oil were obtained; the oil was separated with a few cc. of ether and dried over KOH. This mixture was then fractionated in a 22 cm. microdistilling column as described by Craig (7). After removal of ether, successive fractions were collected under gradually reducing pressure from 36 mm. to 1 mm. and showed the boiling points and analytical results given in Table I.

Further fractionation of this material as such was not attempted, since it appeared less hopeful than isolation as the following methiodide. The formation of the latter in excellent yield from the fractions boiling at 208–209° demonstrated that these fractions consisted essentially of the base  $C_9H_{19}ON$ . The



analytical figures in Table I approximate the calculated values, C 68.72, H 12.18 per cent.

*The Methiodide,  $C_{10}H_{22}ONI$* —0.15 gm. of the above base (b.p. 208°) was treated with excess methyl iodide in ether solution. The methiodide gradually crystallized. After 24 hours 0.28 gm. was collected. On recrystallization from alcohol it formed flat prisms which melted at 242–243°.

$[\alpha]_D^{20} = +14.5^\circ$ ( $c = 1.03$ in 95 per cent alcohol)		
$C_{10}H_{22}ONI$ . Calculated.	C 40.12, H 7.41, CH <sub>3</sub> 10.03	
Found.	" 40.24, " 7.27, " 10.32	

The Tschugaeff-Zerewitinoff determination showed the presence of 1 active H atom due to an OH group. Calculated, 1H 0.34; found, 0.34.

*The Methiodide,  $C_{11}H_{24}ONI$* —0.7 gm. of the above methiodide was suspended in 2 cc. of methyl alcohol and decomposed by intimately mixing with a suspension in alcohol of silver oxide which had been freshly prepared from 0.47 gm. of silver nitrate. AgI was centrifuged off and washed by suspension in a small volume of alcohol and recentrifugation. The clear solution of the ammonium hydroxide which was free from Ag and I ions was transferred to a microdistillation apparatus. Methyl alcohol was carefully removed at barometric pressure. A colorless, viscous, basic oil remained. The pressure was then reduced to 20 mm. and when the oil bath was heated to about 80° decomposition began. By slowly raising the temperature to 90° practically all was distilled and a turbid distillate collected in the condenser cup. The bath was raised finally to 120° to complete the distillation. Only a trace of undistilled residue remained.

The distillate, which consisted of the tertiary dimethyl base and water, was dissolved in about 10 cc. of dry ether and an excess of methyl iodide was added to the solution. A turbidity followed by prompt crystallization of the trimethyl ammonium iodide occurred which became copious on standing. After 24 hours the quaternary salt was collected with ether. It was recrystallized by addition of ether to the solution in methyl alcohol and formed colorless needles which melted at 132–133°. It was very soluble in water and the alcohols.

$[\alpha]_D^{20} = -16^\circ$ ( $c = 1.0$ in water)		
$C_{11}H_{24}ONI$ . Calculated,	C 42.16, H 7.72; found, C 42.27, H 7.56	

In the Tschugaeff-Zerewitinoff determination of active hydrogen, the results of a number of determinations were consistent in giving roughly 0.5 mole of methane. Calculated, 1H 0.32; found, 0.14, 0.17, 0.20.

*Hydrogenation of Cevine*—Although cevine was recovered unchanged following attempts to hydrogenate it with the platinum oxide catalyst of Adams and Shriner, it was found to absorb hydrogen with Raney's nickel catalyst.

A solution of 0.5 gm. of cevine in 3 cc. of methyl alcohol was shaken with an excess of Raney's catalyst suspended in the same solvent. After 24 hours absorption had stopped and 25 cc. of  $H_2$  or roughly 1 mole had been consumed.

After removal of the catalyst the concentrated solution deposited crystals which were recrystallized from methyl alcohol. It contained the solvent of crystallization and softened with effervescence at  $220^\circ$  due to loss of the solvent, but resolidified and then melted at  $263\text{--}265^\circ$ .  $[\alpha]_D^{25} = -8^\circ$  ( $c = 0.96$  in methyl alcohol). For analysis it was dried at  $140^\circ$  under reduced pressure.

$C_{27}H_{46}O_2N \cdot 2CH_3OH$ . Calculated,  $CH_3OH$  11.12; found, 10.46  
 $C_{27}H_{46}O_2N$ . Calculated, C 63.36, H 8.87; found, C 63.44, H 8.98

The substance separated also from chloroform with the solvent of crystallization which was held quite tenaciously when we attempted to dry the substance for analysis. Chloroform was retained even at  $140^\circ$ .

*Reduction of Cevine with Sodium*—3 gm. of cevine were dissolved in 80 cc. of butyl alcohol and heated to boiling. 4 gm. of sodium were then added and the mixture was vigorously shaken to emulsify the sodium. After solution of the metal, water was added and the mixture was concentrated *in vacuo* to remove the butyl alcohol. The aqueous solution was warmed and extracted with chloroform. The latter after being washed and dried over  $K_2CO_3$  was concentrated to dryness. The residue weighed 2.6 gm. It was crystallized from methyl alcohol, forming microplatelets which after recrystallization melted at  $263\text{--}264^\circ$  after preliminary sintering and contained the solvent.  $[\alpha]_D^{25} = -27^\circ$  ( $c = 1.12$  in methyl alcohol). For analysis it was dried at  $140^\circ$  and 20 mm.

In later experiments it was found that if the crude reaction product is first dissolved in a small volume of dry chloroform, fine needles of a sparingly soluble by-product separated in small yield. After collection with chloroform 0.17 gm. was obtained from 3 gm. of cevine. It formed needles from methyl alcohol which melted at 284–287°.

$$[\alpha]_D^{25} = -21^{\circ} \text{ (} c = 1.3 \text{ in methyl alcohol)}$$

$C_{27}H_{43}O_7N$ . Calculated. C 65.67, H 8.79

$C_{27}H_{43}O_7N$ . Calculated, C 65.41, H 9.16; found, C 65.17, H 8.99

In other experiments in which butyl alcohol was replaced by ethyl alcohol as a solvent, practically identical results were obtained.

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## THE UTILIZATION OF PYRUVIC ACID BY BAKERS' YEAST\*

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If sodium pyruvate is added to cells of bakers' yeast suspended in a phosphate buffer of pH 7.0 in the strict absence of oxygen, practically no pyruvate is utilized. After 3 hours in contact with the cells the sodium pyruvate can be almost quantitatively recovered. If, however, oxygen is admitted to the cells, then both pyruvate and oxygen are utilized at appreciable rates (see Table I). So far as I am aware, no one has pointed out this difference between aerobic and anaerobic conditions in connection with pyruvate metabolism by yeast, although one can infer from the literature that such must be the case. Thus Hägglund and Augustsson (1) and Haehn and Glaubitz (2) both remark that the utilization of pyruvate by yeast cells at neutrality is practically nil, whereas Lieben (3) measured a definite utilization of sodium pyruvate by yeast cells while bubbling air through the suspension, and Meyerhof (4) obtained a comparatively large increase in oxygen consumption on adding sodium pyruvate to yeast cells. Such a marked difference between anaerobic and aerobic conditions is rather surprising, for, as is well known, yeast contains an enzyme system, the carboxylase system, that rapidly attacks pyruvic acid under strictly anaerobic, as well as under aerobic conditions, according to the equation,  $\text{CH}_3\text{COCOOH} \rightarrow \text{CH}_3\text{CHO} + \text{CO}_2$ . This enzyme-catalyzed reaction occurs most rapidly at slightly acid reactions, so it might be considered that in experiments at neutrality this enzyme is inhibited. However,

\* A preliminary account of these experiments was presented before the Thirty-second annual meeting of the American Society of Biological Chemists at Baltimore, 1938 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. cxi (1938)).

if the pH is lowered to 5.6 by means of an acetate buffer, isolated carboxylase can be shown to be very active, but living cells are still practically inactive toward pyruvate under anaerobic conditions. In the presence of oxygen, however, they utilize pyruvate and consume even more oxygen than in phosphate buffer at pH 7.0. Apparently under anaerobic conditions the pyruvate cannot come into contact with the carboxylase system in the cell. Under aerobic conditions, however, the pyruvate apparently has ready access to the oxidizing enzyme system.

If the medium is made still more acid, the cells begin to utilize pyruvate at an appreciable rate under anaerobic conditions in accordance with the results of Hägglund and Augustsson (Table I). However, the oxygen consumption under aerobic conditions decreases. The limiting condition employed was to suspend the cells in dilute free pyruvic acid. Under this condition the anaerobic reaction is quite rapid. If we express the amount of pyruvic acid used in terms of the c.mm. of  $\text{CO}_2$  that would be formed from it by carboxylase (*i.e.* 1 gm. molecule of pyruvic acid = 22,400 c.mm.), then the c.mm. of pyruvic acid used per mg. of dry weight of cells per hour ( $Q_{\text{pyruvic acid}}^{\text{N}_2}$ ) reach values of 22 to 24. Under aerobic conditions the c.mm. of oxygen consumed per mg. of dry weight of cells per hour ( $Q_{\text{O}_2}$ ) reach values of this same order of magnitude. If the pyruvic acid is half neutralized, the  $Q_{\text{pyruvic acid}}^{\text{N}_2}$  drops to about one-half the previous value and the  $Q_{\text{O}_2}$  increases about 50 per cent. If the pyruvic acid utilized aerobically,  $Q_{\text{pyruvic acid}}^{\text{O}_2}$ , is determined, it is found to be greater than  $Q_{\text{pyruvic acid}}^{\text{N}_2}$  under all conditions (see Table I) and to vary less with pH. Thus, in free pyruvic acid  $Q_{\text{pyruvic acid}}^{\text{O}_2}$  is about twice  $Q_{\text{pyruvic acid}}^{\text{N}_2}$  but in half neutralized pyruvic acid it may be 4 or more times as great. However, the rate at which pyruvate is utilized also depends on factors other than pH. Thus the addition of urea in a concentration equivalent to that of the free pyruvic acid used has only a very slight effect on the pH but it may increase the rate of pyruvate utilization 3-fold. Various amino acids have a similar effect. They, of course, affect the pH, but their effect on pyruvate utilization is greater than the same change in pH brought about by NaOH. The results mentioned appear to be best explained on the assumption that the limiting factor in the utilization of pyruvate by living yeast cells is the permeability of the cell membrane. Thus, under

anaerobic conditions this membrane would have to be considered as practically impermeable to the pyruvate ion, but permeable to undissociated pyruvic acid. The fact that the rate of utilization may be influenced by the presence of a substance such as urea, a substance which is not attacked by yeast cells under the conditions given, could then be explained as an effect upon the permeability of the membrane. Such an explanation would, of course, mean that the membrane is more permeable to pyruvic acid under aerobic than under anaerobic conditions. A similar change in permeability for glucose, although in the opposite direction, *i.e.* greater permeability under anaerobic conditions, has been postulated by Dixon and Holmes (5) as an explanation of the Pasteur effect. The results of Runnström (6) *et al.* can be interpreted as supporting the theory of Dixon and Holmes. It is, of course, possible that if the permeability of the membrane changes at all on changing conditions, it might change in opposite directions for different substrates.

Although the above considerations must be borne in mind in work with pyruvic acid and yeast cells, the purpose of the present paper is not to consider them further, but to consider the question of what becomes of the pyruvic acid that is utilized under aerobic conditions by cells of bakers' and torula yeast in acid solutions.

Neuberg and von May (7) obtained satisfactory balances for the utilization of pyruvic acid by brewers' yeast. The products were  $\text{CO}_2$ , acetaldehyde, and acetoin. Lieben (3) in the work mentioned above, which was carried out under aerobic conditions but without measurement of the oxygen consumption, found that the  $\text{CO}_2$  produced in his experiments was not sufficient to account for the pyruvate used. He could find no acetaldehyde, so he was left with a negative carbon balance. He found, however, that his cells had increased in weight and if he assumed that the increase in weight was due to carbohydrate formation then the carbon contained in it completed his carbon balance. He did not, however, demonstrate a formation of carbohydrate. Meyerhof (4) in the paper cited above reports only values for oxygen consumption in the case of pyruvic acid. He considers this acid as one of the substances that are converted to carbohydrate by yeast, but his paper gives no direct evidence for it. It appears that not only is direct evidence for such a transformation lacking,

but even simultaneous measurements of oxygen consumption,  $\text{CO}_2$  production, and pyruvate utilization by yeast are lacking. The experiments presented below provide this information.

#### EXPERIMENTAL

The yeast used in most of these experiments was Fleischmann's commercial bakers' yeast purchased in small cakes at a grocery store. It was suspended in distilled water and centrifuged slowly to remove the heavier particles present. This sediment was discarded and the tube then centrifuged rapidly enough to sediment the cells. The supernatant was discarded. This process was repeated three times. The cells were then suspended in either saline or the appropriate salt mixture. Such cells suspended in  $\text{m}/15 \text{ KH}_2\text{PO}_4$  containing 3 per cent glucose in an atmosphere of nitrogen and shaken in the usual manometric apparatus at  $28^\circ$  produced  $\text{CO}_2$  at the rate of 210 c.mm. per mg. of dry weight of cells per hour ( $Q_{\text{CO}_2}^{\text{N}_2} = 210$ ). If suspended in an atmosphere of oxygen, they consume oxygen at the rate of 65 c.mm. per mg. per hour ( $Q_{\text{O}_2}^{\text{O}_2} = 65$ ) and produce  $\text{CO}_2$  at the rate of 112 c.mm. per mg. per hour. If we assume an R.Q. of 1.00, this gives an aerobic fermentation of 47 c.mm. of  $\text{CO}_2$  per mg. per hour ( $Q_{\text{CO}_2}^{\text{O}_2} = 47$ ). If the cells are suspended in  $0.02 \text{ M HCl}$ , the  $Q_{\text{CO}_2}^{\text{N}_2} = 87$ , the  $Q_{\text{O}_2}^{\text{O}_2} = 31$ , and the  $Q_{\text{CO}_2}^{\text{O}_2} = 30$ . Some experiments were also performed with a torula yeast<sup>1</sup> cultivated in this laboratory on unhopped malt. In contrast to the torula yeasts studied by Meyerhof (4) this yeast did not have a high oxygen consumption. The cells suspended in  $\text{m}/15 \text{ KH}_2\text{PO}_4$  containing 3 per cent glucose had a  $Q_{\text{CO}_2}^{\text{N}_2} = 193$ , a  $Q_{\text{O}_2}^{\text{O}_2} = 44$ , and a  $Q_{\text{CO}_2}^{\text{O}_2} = 123$ .

Free pyruvic acid is somewhat toxic to yeast cells, as has been pointed out by Haehn and Glaubitz, so its concentration must be kept low. We have found that cells may be suspended in  $0.08 \text{ M}$  pyruvic acid and allowed to remain there for 3 hours at  $28^\circ$  without being damaged, if we take the property of staining with methylene blue as the criterion of damage. The pH in such a solution as determined by the glass electrode is approximately 2.0. If the pyruvic acid is half neutralized by  $\text{NaOH}$ , the pH is 2.65.

<sup>1</sup> The culture of this yeast was very kindly supplied to us by the Wallerstein Laboratories in New York.

An equimolecular mixture of pyruvic acid and glycine has a pH of 2.55. Since acid is destroyed during the experiment, the solutions become less acid. If a sufficient amount of the solution is taken and the experiment stopped within 3 hours, the pH change is small and may be neglected.

Table I shows the amounts of pyruvic acid used under anaerobic and aerobic conditions in a number of experiments. The amount of cells used varied from one experiment to another. Pyruvic acid was determined by means of carboxylase. As carboxylase we used either a product prepared according to Axmacher and

TABLE I  
*Pyruvic Acid Used Expressed As Difference in C.Mm. of CO<sub>2</sub> Produced by Carboxylase before and after Experiment. Temperature = 28°*

Yeast used	pH	Time	Anaerobically	Aerobically	Yeast used	pH	Time	Anaerobically	Aerobically
		min.					min.		
Bakers'	7.0	180	14	121	Bakers'	2.5	120	421	920
"	5.6	180	16	79	"	2.5	120	450	980
"	5.6	150	15		"	2.5	140	255	720
"	4.6	150	64		"	2.55	60	580	1550
"	3.6	150	127		"	2.65	60	150	413
"	2.0	60	360	606	"	2.65	60	161	670
"	2.0	60	533	1260	"	2.65	60	220	470
"	2.5	60	150	691	"	2.65	220	264	912
"	2.5	50	381	1282	Torula	2.5	50	183	227
"	2.5	60	195	475	"	2.5	55	192	260
"	2.5	90	362	900	"	2.5	120	194	286

Bergstermann (8) or a dialyzed yeast extract, as described by Warburg and Christian (9). The amount of pyruvic acid used was determined by running carboxylase estimations on the initial and the final solutions. The CO<sub>2</sub> formed in these determinations was measured manometrically.

Table II presents a number of experiments showing along with other data the amount of pyruvic acid used, the amount of oxygen used, and the amount of CO<sub>2</sub> produced. The amount of cells used in the experiments in Table II was approximately 25 mg., dry weight, but the different experiments varied around this figure. It may be observed that the amounts of CO<sub>2</sub> produced are large in com-



TABLE II  
*Balance of Pyruvic Acid and O<sub>2</sub> Used and Acetaldehyde and CO<sub>2</sub> Produced*

Experiment No.	Yeast used	Yeast and pyruvic acid						Controls, yeast and HCl			
		Medium	Pyruvic acid used c.mm.*	O <sub>2</sub> used c.mm.	CO <sub>2</sub> produced c.mm.	Acetaldehyde produced c.mm.*	R.Q.†	O <sub>2</sub> used c.mm.	CO <sub>2</sub> produced c.mm.	R.Q.	R.Q.†
1	Bakers'	Free pyruvic acid	937	1184	2340	76	1.18	435	411	0.94	1.32
2	"	"	980	1095	2338		1.24	438	423	0.96	1.40
3	"	"	1108	1496	2962		1.20	578	572	0.99	1.33
4	"	"	1246	1376	2892		1.20	680	689	1.01	1.38
5	"	"	746	570	1820	244	1.88				
6	"	"	1166	593	1852		1.16				
7	"	"	1508	1786	3298	None	1.01				
8 (1 hr.)	"	"	612	565	1320	126	1.25				
(2 hrs.)	"	"	960	919	2061	88	1.20				
(3 " )	"	"	1128	1135	2623	86	1.31				
9 (1 hr.)	"	"	725	611	1523		1.31	295	271	0.92	1.67
(2 hrs.)	"	"	1140	1114	2290		1.03	432	384	0.89	1.12
(3 " )	"	"	1400	1598	3325		1.20	544	504	0.92	1.35
10	Torula	"	380	192	660	70	1.46	107	104	0.97	2.07
11	"	"	930	822	1815		1.08				
12	Bakers'	Pyruvic acid + 0.87 equivalent glycine	3051	1835	5334	629	1.25				

13	Bakers'	Pyruvic acid + 1.0 equivalent glycine	1560	1124	3289		1.54	252	238	0.94	1.71
14	"	"	1770	912	2772	220	1.10	242			
15	"	"	1460	672	2446	384	1.47		210	0.87	1.18
16	"	Pyruvic acid + 0.5 equivalent NaOH	470	1160	1500		0.89				
17	"	"	1148	1521	3253	284	1.39				
18	"	Pyruvic acid + 0.5 equivalent $\text{Na}_2\text{HPO}_4$	1260	698	2277		1.46				

\* 1 Gm. molecule = 22,400 c.mm.

† R.Q.' =  $\frac{\text{c.mm. of CO}_2 \text{ produced} - \text{c.mm. of pyruvic acid used}}{\text{c.mm. of O}_2 \text{ used}}$ .

‡ R.Q.'' = R.Q.' corrected for the  $\text{O}_2$  used and  $\text{CO}_2$  produced by the control.

parison with the amounts of oxygen used. Such a comparison means very little in this case, for one cannot consider this to be the R.Q. of the pyruvic acid. This is true because yeast can form  $\text{CO}_2$  from pyruvic acid anaerobically according to the equation given at the beginning of this paper. This anaerobic reaction may also occur in the presence of oxygen and the  $\text{CO}_2$  produced by it cannot be distinguished from that produced oxidatively. It seems safest for the present purpose to assume that each molecule of pyruvic acid used produces 1 molecule of  $\text{CO}_2$  anaerobically. The product resulting from this reaction is acetaldehyde. The complete oxidation of acetaldehyde yields an R.Q. of 0.8. Theoretically then in such an experiment if the c.mm. of pyruvic acid used are subtracted from the c.mm. of  $\text{CO}_2$  produced and the resultant number is divided by the c.mm. of oxygen used, the quotient, designated as R.Q.', should be 0.8, or less if the oxidation is incomplete. As shown in Table II it is in every case considerably greater than 0.8. The average for all the experiments shown in Table II is 1.31. Such a marked discrepancy can only be explained as the result of some additional reaction.

The figures used in computing R.Q.' were not corrected for the oxygen consumed and  $\text{CO}_2$  produced by the controls. As a control, we understand a similar suspension of cells brought to the same pH as in the pyruvic acid experiment by the addition of HCl and treated in exactly the same way, but without the addition of any substrate. The values observed for such controls are also shown in Table II. It may be observed that with one exception the R.Q. of these controls is less than 1.00. If the experiments are corrected for these controls, the effect in every case is to raise the R.Q.' higher. These corrected figures, R.Q.", are shown in the last column of Table II. The average R.Q." is 1.45.

In experiments such as these it often is not clear whether or not the experimental figures should be corrected for the controls. These control figures represent an endogenous metabolism and it is usually doubtful whether this continues after the addition of a substrate. In this case the control figures (R.Q. = 0.94) appear to represent a carbohydrate oxidation. It seemed it should be possible to determine whether or not this was actually a carbohydrate utilization and whether or not it continued in the presence of pyruvic acid. A number of carbohydrate determinations have

accordingly been carried out and the results are presented in Table III.

These determinations were carried out by hydrolyzing the cells with 1.0 N  $\text{H}_2\text{SO}_4$  for 3 hours at  $100^\circ$ . Reducing sugars were then estimated by the method of Hagedorn and Jensen (10) as modified for larger amounts by Hanes (11). The proteins were precipitated by  $\text{Zn}(\text{OH})_2$  and separated by centrifuging instead of filtering. In some cases fermentable sugar was also determined. In these cases the  $\text{H}_2\text{SO}_4$  hydrolysate was neutralized with  $\text{NaOH}$  and the fermentation carried out without precipitating the proteins. The two methods do not give the same results. The value for reducing sugar runs about 11 per cent higher. However,

TABLE III  
*Hydrolyzable Carbohydrate As Mg. of Glucose*

Time	Control at start	HCl cells	Pyruvic acid cells	$\text{KH}_2\text{PO}_4$ cells
<i>hrs.</i>				
3.0	8.30	6.65	6.24	5.54
2.0	8.69	7.25	7.14	
2.5	8.38	7.76	7.49	
3.0	6.85	5.05		
2.0	8.56		7.76	
3.0	8.56		7.40	
1.0	6.83	6.33	6.22	
3.0	6.83		4.67	

since the values desired were always differences from controls treated in the same way, either method may be used.

The results show quite clearly that the carbohydrate determined in this way does decrease as the cells are shaken in 0.02 N HCl. In fact the decrease obtained by this analysis is always greater than the measured oxygen consumption or  $\text{CO}_2$  production would indicate. In some cases it was twice as great. The explanation for this is not clear. It may be that some carbohydrate passes out from the cells into the HCl, but analyses on the HCl for reducing sugars as well as for nitrogen were both negative. It seems more likely that while the cells were shaken with 0.02 N HCl intermediate carbohydrate products were formed within the cell which were destroyed by heating with  $\text{H}_2\text{SO}_4$ . Table III

also shows that the carbohydrate in the cells which were shaken with pyruvic acid decreased as much as or even a little more than in the cells shaken with 0.02 N HCl. According to these results the endogenous metabolism of the controls continued after the addition of pyruvic acid, so to find the gas exchange due to the addition of pyruvic acid the figures obtained should be corrected for the controls. This leaves us with the task of explaining an R.Q. of 1.45 instead of the theoretical 0.8.

It would seem that the only possible explanation for such a high R.Q. is that some reduction product is formed. Before the reduction level of such a product can be estimated, it is necessary to know how much acetaldehyde is present at the end of the experiment. This has been determined in a number of the experiments by aerating it into bisulfite in the lactic acid apparatus of Wendel (12) and titrating with iodine. The values obtained are recorded in Table II. If we now consider a balance for Experiment 1 in Table II, correcting for the control and considering all substances as gases so that they may be directly compared, *i.e.* considering 1 gm. molecule of each substance equal to 22,400 c.mm., we find that 937 c.mm. of pyruvic acid + 749 c.mm. of  $O_2$  have yielded 1929 c.mm. of  $CO_2$  + 76 c.mm. of acetaldehyde. Part of the  $CO_2$  was probably formed anaerobically by decarboxylation of pyruvic acid and the rest was formed oxidatively. Of the  $(3 \times 937)$  c.mm. of carbon contained in the pyruvic acid used  $(1929 + (2 \times 76))$  c.mm. were recovered in the  $CO_2$  and acetaldehyde produced. This is a little over 74 per cent recovered. Of the  $(1.5 \times 937)$  c.mm. of  $O_2$  contained in the pyruvic acid used and the 749 c.mm. of  $O_2$  absorbed as a gas, the two products considered account for  $(1929 + (0.5 \times 76))$  c.mm. This is a little over 91 per cent recovered. However, of the  $(2 \times 937)$  c.mm. of  $H_2$  contained in the pyruvic acid used only  $(2 \times 76)$  c.mm. are represented in these two products. This is only about 8 per cent recovered. It is obvious that whatever products remain must be strongly reduced substances. In an effort to find out what these were we have carried out the tests described below.

One possibility that suggests itself is that pyruvic acid under aerobic conditions stimulates the fermentation of the reserve carbohydrate of the cell. Table III demonstrates that this reserve carbohydrate decreases to about the same extent as the

control, but it is possible that this material is respired in the control, but fermented in the experiment. Each molecule of glucose would then give rise to 2 molecules each of  $\text{CO}_2$  and ethyl alcohol without using any oxygen. There are two reasons for not considering this further. Firstly, it requires a formation of ethyl alcohol and, as discussed below, this cannot be demonstrated and, secondly, a simple calculation on the data presented in Table II shows that such a consideration, if granted, will not explain the results.

Volatile alcohols were tested for in the following way. If the acetaldehyde determination described above is carried out with a well cooled condenser, substances less volatile than acetaldehyde may be retained. If the solution is then distilled, these products will pass into the distillate and can be determined there. Such distillates in most of our experiments contained nothing that reacts with a strongly alkaline iodine solution or with hot  $\text{KMnO}_4$ . The solutions were neutralized with  $\text{NaOH}$  before distillation in order to prevent the distillation of the pyruvic acid. The small amounts of material present in some distillates could be accounted for as acetoin according to the method of Langlykke and Peterson (13). The values obtained were of the order of one-tenth of the acetaldehyde values and they will not be considered further here. As a check, however, solutions were distilled without previously removing the acetaldehyde and the distillates were treated with alkaline iodine. The first quarter of the distillate contains all the acetaldehyde and from the fact that the figures obtained agree with the figures for acetaldehyde obtained by the bisulfite method we conclude that it contained no other products such as ethyl alcohol or acetone.

Lactic acid determinations have been run according to Friedemann, Cotonio, and Shaffer (14) and none was found. In fact, no acid other than pyruvic is present in the solution at the end of the experiment. This can be shown in the experiments with free pyruvic acid by comparing carboxylase determinations with the titration of the total acid. For example, in one experiment 1488 c.mm. of pyruvic acid were used according to the carboxylase determinations, and titration to phenolphthalein with  $\text{NaOH}$  indicated a total acid disappearance of 1534 c.mm.

Glycerol determinations were carried out on the solution ac-

according to Zeisel and Fanto (15). Traces appear to be present, but the amount is very small.

Although yeast is not known to produce hydrogen gas or methane gas, we have nevertheless tested for their presence. Two methods were used for hydrogen. In the first the gas mixture at the end of the experiment was shaken with alkaline pyrogallol and then brought into contact with a platinized platinum electrode that dipped into an HCl solution. If even very small amounts of hydrogen are present, and oxygen is absent, such an electrode assumes a strongly negative potential. In these experiments no evidence for the presence of hydrogen could be obtained. The other method was to measure the volume of gas remaining after the mixture of gases was shaken with alkaline pyrogallol. If the experiment is started with pure oxygen and no gas other than carbon dioxide is produced, then the gas should be completely soluble in alkaline pyrogallol both at the beginning and at the end of the experiment. Actually the commercial oxygen used contains about 1.5 per cent of a gas not soluble in this reagent. No additional insoluble gas was produced by the experiment.

It was, of course, obvious from the beginning that the product formed might remain inside the cells. Attempts to demonstrate an increase in weight of the cells were not successful. The results indicate a small decrease in weight, but such experiments are subject to considerable error. In considering what product might be formed inside the cell we must consider the possibility that a carbohydrate is formed that is not hydrolyzed by the method employed here. That this cannot be a sufficient explanation is clear from the fact that such a transformation of pyruvic acid to carbohydrate is only a very slight reduction. Thus for every 6 carbon atoms converted to carbohydrate only the equivalent of 1 oxygen atom is formed. If all the carbon unaccounted for in Experiment 1 of Table II were so converted, the balance with respect to oxidation and reduction would still be far from complete. A more promising explanation would be the conversion of part of the pyruvic acid to fat. This is an enormous reduction—the conversion of 1.0 mg. of pyruvic acid to a saturated  $C_{18}$  fatty acid liberates the equivalent of 466 c.mm. of oxygen. The conversion of carbohydrate to fat is of course the classical explanation for a high R.Q. Furthermore, the conditions stated by Maclean

(16) to be favorable for fat formation in yeast cells are a free supply of oxygen and a non-nitrogenous medium rich in carbohydrate. Others (17) have stated that an acid medium is favorable. These conditions are more or less fulfilled by our experiments. It is interesting to note that Maclean remarked that the part played by oxygen requires further elucidation. At first thought it may seem strange that oxygen should be required

TABLE IV

*Ether Extract in Mg. from 1.95 to 1.50 Gm. (Dry Weight) Samples of Control and Pyruvic Acid-Treated Cells*

Heated with acid			Heated with alkali		
Experiment No.	Control, HCl cells	Pyruvic acid cells	Experiment No.	Control, HCl cells	Pyruvic acid cells
1	107	123	11	169	203
2	112	157	12	142	170
3	94	108	13	140	151
4	74	87	14	129	136
5	58	97	15	124	138
6	84*	112*	16	98	106
7	82*	96*	17	96	109
	(47)	(46)	18	79	103
8	73*	78*			
	(24)	(23)			
9	51*	61*			
	(18)	(23)			
10	64*	76*			
	(33)	(41)			

The figures given in parentheses represent the material extracted by ethyl ether, but insoluble, or at least not readily soluble, in petroleum ether.

\* Petroleum ether-soluble.

to produce such a highly reduced substance as a fat. If we remember, however, that the formation of fat represents a storage of energy, then it seems very probable that the rôle of oxygen is to bring about reactions supplying the necessary amount of energy.

Determinations of the amount of material extractable by ether in the cells have been carried out and the results are presented in Table IV. As discussed by Maclean (16) and others, only a small amount of the fat present can be extracted from yeast



cells by organic solvents, but if the cells are first heated with acid the fat can then be extracted. We have accordingly followed this method. The well washed cells were heated with 1.0 N HCl for 2 hours at 100°. This solution was then evaporated to dryness on a water bath, and the dry residue transferred to an extraction thimble, placed in a Soxhlet apparatus, and extracted with ethyl ether. Extraction was continued in all cases for 48 hours and in some for 96 hours. A typical example of the amount of extract obtained at different times is the following: first 24 hours 58 mg., second 24 hours 7 mg., third 24 hours 4 mg. As shown in the first five experiments of Table IV, this method indicates that cells shaken with pyruvic acid contain more material extractable by ether than the controls which had been shaken with HCl. It must, of course, be made clear that this increase is not pyruvic acid. In order to remove all pyruvic acid on the outside of the cells they were washed three times in 50 times their volume of water. The controls were, of course, washed in the same way. It is conceivable that pyruvic acid might accumulate inside the cell in spite of the presence of carboxylase, although such an accumulation would not fit well with the other data. We have, accordingly, tested the extract for pyruvic and other keto acids by evaporating off the ether (*in vacuo*, under CO<sub>2</sub>), extracting the residue with water, and testing the water with carboxylase and with phenylhydrazine. No evidence for keto acids was obtained.

In Experiments 6, 7, 8, 9, and 10 of Table IV the dried ether extract was extracted again with petroleum ether. It is well known that ethyl ether dissolves things other than fats and fatty acids and that petroleum ether is supposed to be a more specific solvent for fatty acids. It may be observed that the petroleum ether-soluble fraction is greater in the case of the pyruvic acid cells than it is in the control. The figures given in parentheses for Experiments 7, 8, 9, and 10 represent the material extracted by ethyl ether, but insoluble, or at least not readily soluble, in petroleum ether.

Experiments 11 to 18 inclusive of Table IV were carried out in a different way. In these the washed cells were treated with alcoholic KOH in such quantities that the final concentration of alkali was 1.0 N. They were then refluxed for 4 hours, acidified

with  $\text{H}_2\text{SO}_4$ , and extracted three times with ethyl ether. It may be seen that here again the values for the pyruvic acid cells exceed the controls. These figures were obtained from amounts of bakers' yeast varying from 1.25 to 1.50 gm., dry weight. The individual experiments are not to be compared with one another, but each pyruvic acid experiment is to be compared with its own control.

It seems clear from these results that the ether extract of yeast cells is increased by shaking them with pyruvic acid under aerobic conditions. From the fact that this increase is soluble in petroleum ether we may consider it, at least temporarily, as fat or fatty acid and see whether such a formation of fat will explain our other results. As stated above, these figures were obtained from experiments with larger amounts of yeast and cannot be directly compared with the experiments in Table II. It seems certain that the formation of no one substance will account quantitatively for all the results presented in Table II, but qualitatively a formation of fat in the amounts indicated by Table IV will serve to bring all of the experiments into something approaching an oxidation-reduction balance.

The figures given do not indicate whether the pyruvic acid itself should be considered as being converted to fat or whether the carbohydrate that disappears, and which is oxidized in the control, is in the presence of pyruvic acid converted to fat and the oxygen which is released used to oxidize the pyruvic acid. If the latter were the case, one might expect that the addition of other readily oxidizable substrates would lead to similar results. This appears not to be the case. For example, if acetaldehyde is added instead of pyruvic acid one finds not a high r.q. but one slightly too low (r.q. = 0.73). This can be readily explained by an incomplete oxidation. Similarly if the cells are suspended in free lactic acid or in a buffered solution of lactate an oxidation readily occurs but the r.q. obtained is the theoretical one of 1.00. The effects described above appear to be specific for pyruvic acid. The task of following the reactions involved is a difficult one if only the usual chemical methods are employed. Possibly the task would yield readily to an attack involving the use of isotopes.

The oxygen consumption due to pyruvic acid at pH 2.5 is readily inhibited by iodoacetate. A concentration of 0.0021 M

iodoacetate decreases the oxygen consumed to one-tenth its former value and decreases the amount of pyruvic acid used to approximately the anaerobic level. The anaerobic reaction is not affected by this concentration of iodoacetate. Iodoacetamide is a much less effective inhibitor.

#### SUMMARY

The utilization of pyruvic acid by cells of bakers' yeast and a torula yeast under anaerobic and aerobic conditions and at different pH values has been measured and discussed.

It is shown that the amount of pyruvic acid used under aerobic conditions is from 2 to 4 or more times greater than the amount used under anaerobic conditions. The amount of oxygen consumed in this aerobic reaction is not sufficient to account for the production of the amount of  $\text{CO}_2$  obtained from the amount of pyruvic acid used.

Examination of the liquid in which the cells were suspended and of the gas in the vessel failed to reveal the presence of any reduction product in significant amounts. Determinations of hydrolyzable carbohydrate in the cells show that this material decreases if cells are shaken in dilute HCl in the presence of oxygen but without added substrate, and that it decreases as rapidly or even more rapidly from cells shaken with dilute pyruvic acid. Thus hydrolyzable carbohydrate is not formed from the pyruvic acid used.

Determinations of ether-extractable material present in cells previously heated with acid or alkali show that this material is increased in cells treated with pyruvic acid as compared to those treated with HCl. The petroleum ether-soluble material present in the ethyl ether extract is increased by about the same amount. Thus fats or substances closely related to the fats are formed from the pyruvic acid.

The utilization of pyruvic acid under aerobic conditions at pH 2.5 is readily decreased to the level attained under anaerobic conditions by low concentrations of iodoacetic acid. It is much less readily inhibited by iodoacetamide.

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## THE B VITAMINS AND FAT METABOLISM

### I. EFFECTS OF THIAMINE, RIBOFLAVIN, AND RICE POLISH CONCENTRATE UPON BODY FAT

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In 1936 Whipple and Church (1) found that the difference in weight caused by thiamine in paired fed rats could be attributed to differences in the amounts of fat and water in the bodies. Since fat-free diets were used, it seemed likely that the increase in fat was due to synthesis from carbohydrate. In the following year one of us reported (2) the apparent production of fat from carbohydrate in the livers of rats fed a fat-free diet containing thiamine and riboflavin but as little choline as possible. Supplee and associates (3, 4) have stated that thiamine, riboflavin, and rice polish concentrate, used as a source of vitamin B<sub>6</sub>, have associated effects on the weight of young rats. Since we believe that one function of thiamine is the formation of fat, it appeared advantageous to investigate whether riboflavin and rice polish concentrate produced changes in weight, partially at least, by influences upon fat metabolism.

#### *Methods*

Young white rats of the Wistar strain, reared in the Connaught Laboratories' colony, were employed. They were kept in individual, screen bottom cages with water available freely. The animals were weighed daily and records were kept of food consumption. Groups of ten rats were used and all the results given are averages for such groups. In some cases the basal diets, supplied *ad libitum*, were similar to that employed by Supplee *et al.*, and in others were modified to contain as small quantities as possible of fat and choline. In Diet 3 casein was reduced to

10 per cent to diminish the lipotropic effect of that substance, originally described by Best and Huntsman (5).

The compositions of the basal diets are given in Table I. The following supplements were used: thiamine (Merck), riboflavin (Hoffmann-La Roche), rice polish concentrate (Labco), and choline (British Drug Houses). Because of the amounts of thiamine and of choline in the rice polish concentrate, it was necessary to consider the effects of choline in conjunction with thiamine as a control upon the effects of rice polish concentrate. Since the amount of free choline supplied was equivalent to the amount in the concentrate (429 mg. per 100 gm.), it was much less than is ordinarily used for studying the effect of choline. In Series I all supplements were provided in such amounts that the quantities

TABLE I  
*Composition of Basal Diets*

Constituent	Diet 1	Diet 2	Diet 3
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein (Labco vitamin-free).....	20	20	10
Sucrose.....	69	74	84
Corn oil (Mazola).....	3	0	0
Salt mixture (Steenbock-Nelson Salts 40 (6)).	4	4	4
Agar.....	2	2	2
Cod liver oil.....	2	0	0
“ “ “ concentrate.....	0	0.015	0.015

of thiamine and choline received by various groups were the same; for example, all rats given thiamine secured the same amount whether or not rice polish concentrate was present. Animals in Series I received amounts of supplements which were independent of the food intake. In other series all supplements were given in proportion to the amount of food consumed. The quantities of supplements are noted in the reports on each series.

After the animals had been killed by stunning, fat determinations were carried out by the Leathes and Raper modification (7) of the Liebermann saponification procedure, with some alterations in technique. The bodies from each group were weighed and placed in a 12 liter, round bottom flask. For each 100 gm. of body weight 50 gm. of potassium hydroxide and 33 cc. of water

were added. The flask was heated in a boiling water bath for 6 hours. 95 per cent ethyl alcohol was added in the proportion of 50 cc. per 100 gm. of body weight. After a reflux condenser was attached, the flask was reheated for 1 hour. The condenser was then removed and heating continued until the alcohol had been evaporated. The total volume of the residue was measured and four 50 cc. portions were transferred to 200 cc. round bottom flasks with extended necks. To each was added sufficient 40 per cent sulfuric acid to make the contents acid to litmus. The flasks were chilled and to each were added 50 cc. of petroleum ether and sufficient water to make the total volume 200 cc. The mixtures were thoroughly shaken and allowed to stand in the refrigerator for 4 hours. From each flask a 10 cc. portion of the petroleum ether layer was transferred to a tared 50 cc. Erlenmeyer flask. The petroleum ether was removed by evaporation, and the flask dried overnight in a desiccator and weighed. The residue is a mixture of fatty acids and unsaponifiable material and the results are given as the percentage of the residue, referred to as total crude fatty acids, in the original body.

#### EXPERIMENTAL

*Series I*—In this series a procedure similar to that reported by Supplee and associates was employed. The animals were maintained on the basal diet for 2 weeks, after which all groups, except one reserved as a control, received supplements for 1 week. Basal Diet 1 was used and the following quantities of supplements were provided in the second period: thiamine, 12.5 micrograms; choline, 1.3 mg.; riboflavin, 10 micrograms; rice polish concentrate, 300 mg. per rat per day.

Fig. 1 shows the curves of average weights of each group of animals and Table II summarizes the weight changes and total food consumption in the 3rd week and gives the results of fat determinations at the end of the experiment.

*Series II*—In this and in Series III there was no depletion period; supplements were given from the start of the experimental feeding. Basal Diet 2 was used. The quantities of supplements reported for Series I were provided in each 10 gm. portion of the diet instead of per rat per day.

Fig. 2 shows the curves of average weights for each group and



Table III summarizes the weight changes and total food consumption and gives the results of fat determinations at the termination of the experiment.

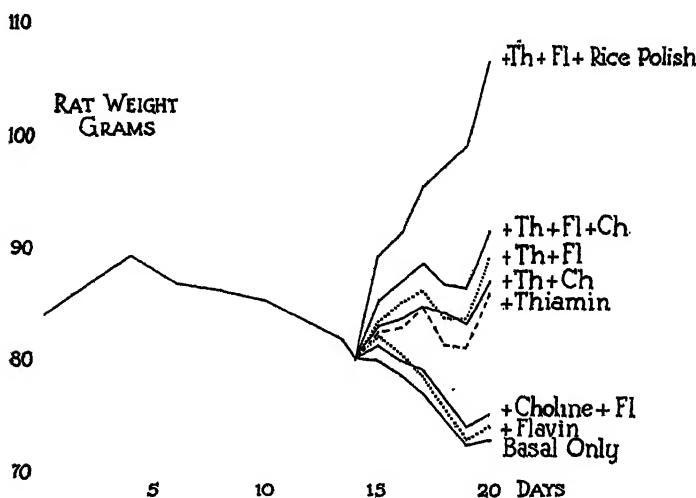


FIG. 1. Curves of average weights for Series I

TABLE II  
of Vitamin B Supplements after Depletion Period

Supplements	Average individual weight changes in 3rd wk.	Average individual food intake in 3rd wk.	Total crude fatty acids
	gm.	gm.	per cent
None, control group.....	-7	35	2.4
Thiamine.....	+6	55	5.3
" + choline.....	+7	51	4.8
" + flavin.....	+9	57	5.8
" + " + choline.....	+12	60	4.6
" + " + rice polish concentrate.....	+26	71	6.3
Flavin.....	-5	32	1.8
" + choline.....	-6	32	1.7

*Series III*—The same procedure as in Series II was used; basal Diet 3 was employed, and the experimental feeding was continued

for 3 weeks instead of 2. Since the weight curves have the same general appearance as for Series II, they will not be reproduced. Table III summarizes the weight changes and total food consumption and the fat content of the bodies at the end of the experiment.

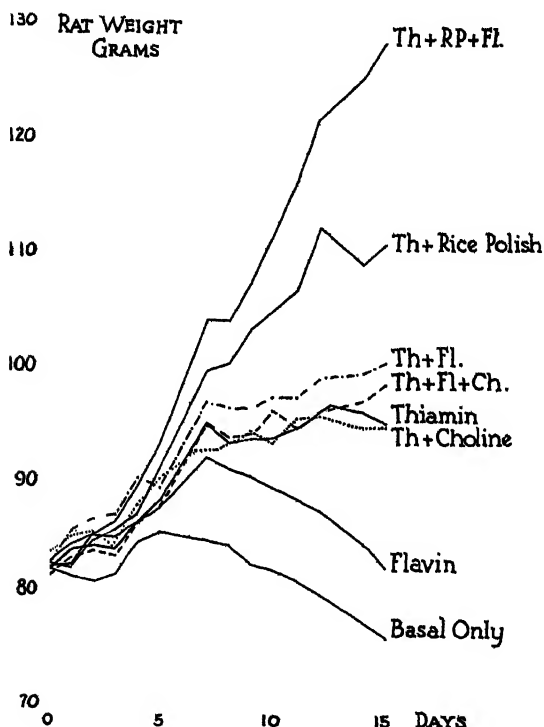


FIG. 2. Curves of average weights for Series II

#### DISCUSSION

Supplee and associates found, as have others, that the addition of riboflavin to a diet deficient in all the B vitamins has little effect upon weight changes. Thiamine causes some increase in weight; this effect is somewhat augmented by the addition of riboflavin. Rice polish concentrate was found by Supplee *et al.* to increase the effect of thiamine; the use of all three supplements gave the greatest increase in weight. These results have been confirmed in the above report.

In the experiments described above and in many others conducted by us thiamine has caused the amount of body fat to be greater than in the unsupplemented controls. This has been interpreted as a demonstration of fat synthesis from carbohydrate, since the diets are practically devoid of fat. Riboflavin, as the only supplement, has consistently produced a reduction in body

TABLE III  
*Effects of Vitamin B Supplements upon Body Weight and Body Fat*

Supplements	Average individual total weight changes	Average individual total food intake	Total crude fatty acids
Series II. Supplemented Diet 2 fed			
None, control group.....	gm. -6	gm. 101	per cent 3.1
Flavin.....	0	115	2.5
Thiamine.....	+13	128	4.9
" + choline.....	+13	118	3.9
" + flavin.....	+18	124	4.7
" + " + choline.....	+16	121	5.5
" + rice polish concentrate.....	+28	130	6.3
" + " " " +			
flavin.....	+46	157	9.2
Series III. Supplemented Diet 3 fed			
None, control group.....	-20	113	2.2
Flavin.....	-14	141	1.9
Thiamine.....	+9	176	4.8
" + choline.....	+11	182	5.0
" + flavin.....	+10	178	3.8
" + " + choline.....	+11	180	5.3
" + rice polish concentrate.....	+26	210	7.4
" + " " " +			
flavin.....	+42	250	11.2

fat, although the difference is slight. In conjunction with thiamine, neither riboflavin, choline, nor the two together have produced any appreciable change in body fat in comparison with animals receiving thiamine only. Rice polish concentrate definitely increases the amount of body fat and this is augmented by the addition of riboflavin. Since other groups received the same

amounts of thiamine and of choline as are present in rice polish concentrate, the effect of the concentrate is not due to these constituents. Supplee *et al.* have attributed the influence of rice polish concentrate upon weight to its content of vitamin B<sub>6</sub>, since the weight changes could be correlated to the absence or the presence of dermatitis. The concentrate is a crude preparation and we refrain from drawing the conclusion that its effect upon body fat is due to that vitamin. It is of interest that Halliday (8) has found that preparations of vitamin B<sub>6</sub> made from liver cause a reduction in liver fat, presumably by transport of fat to the

TABLE IV  
*Relative Changes in Crude Fatty Acids, Body Weight, and Food Intake*

Supplements	Total crude fatty acids	Ratios of crude fatty acids*	Ratios of final weights*	Ratios of food intake*
	gm.			
None, control group.....	2.4	1.0	1.0	1.0
Flavin.....	2.1	0.9	1.1	1.1
Thiamine.....	4.7	1.9	1.2	1.3
“ + choline.....	3.7	1.5	1.2	1.2
“ + flavin.....	4.7	1.9	1.3	1.2
“ + “ + choline.....	5.4	2.2	1.3	1.2
“ + rice polish concentrate.....	6.9	2.9	1.4	1.3
Thiamine + rice polish concentrate + flavin.....	11.8	5.0	1.7	1.6

\* In each case the value of the control group is taken as unity.

depots. We are indebted to Dr. Halliday for allowing us to see her paper before publication.

In the attempt to correlate the weight changes with alterations in body fat, it can be said that there appear to be parallel changes in body weight and in body fat but the increases in fat only partially account for the total weight changes.

Examination of the food intakes would lead to the conclusion that the supplements increase food consumption. So far as thiamine is concerned Whipple and Church and one of us have reported that thiamine causes an increase in weight and in body fat in paired feeding experiments. When rats are allowed to eat freely, there is an increased effect of thiamine due to appetite

stimulation. We believe that the same can be said for the other supplements and that part of the effects reported in this paper are due to increases in food consumption. Calculations of the ratios of total crude fatty acids, body weights, and food intakes, the value of the control group being taken as unity in each case, have been made. The results for Series II are reported in Table IV. The results for the other series are entirely similar but are omitted for brevity. These ratios indicate that there is parallelism between body weight and food intake but not between these two and body fat. This is increased entirely out of proportion to the food intake.

In studies upon fat movement and fat metabolism a number of dietary constituents may influence the results. Since the effects of some are complementary and of others antagonistic in laying down body fat, the final results will depend upon the relative proportions of the various factors. The results which we have reported were complicated by the lipotropic action of dietary casein which encouraged an increase in body fat. Not only will the amount of casein influence the result but different caseins have different lipotropic effects. The quantity and kind of casein used should be considered in work of this nature upon fat metabolism.

#### SUMMARY

In confirming the reports of Supplee *et al.* that thiamine, riboflavin, and rice polish concentrate have associated effects upon the body weights of young rats, it has been found that these supplements have associated effects upon the amount of body fat, partially accounting for the increases in body weight.

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# THE PROTAMINE SALTS OF PHOSPHATIDES, WITH REMARKS ON THE PROBLEM OF LIPOPROTEINS\*

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In the course of a study of the rôle of protamines in blood coagulation, to be described in the paper immediately following (1), it was observed that a stable emulsion of highly purified cephalin gave a characteristic precipitate on addition of a solution of the protamine salmine. In contrast, neither lecithin nor sphingomyelin produced precipitates under similar conditions. This reaction which seemed to offer a convenient model for the formation of complexes between phosphatides and proteins warranted a closer investigation. That the precipitation of cephalin was not a simple flocculation due to a salt effect could be immediately seen from the fact that the isolated precipitates could not be reemulsified in water. On drying, powders of high nitrogen content were obtained ( $P:N = 1:4$  or  $1:5$ ) which in contact with water swelled to form a rubber-like elastic mass. The products were soluble in organic solvents, could be recrystallized unaltered from ethyl acetate, and did not change their composition following treatment of their solutions in ether with dilute acids or reprecipitation with acetone.

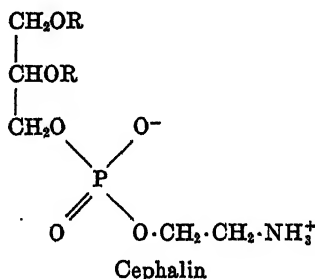
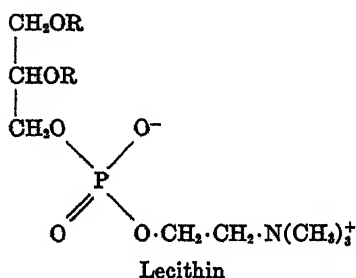
From the behavior of the cephalin-salmine compounds it may be concluded that they are not simple adsorbates, but water-insoluble salts between the acidic cephalin and the strongly basic salmine. A study of the influence exerted by buffers of varying pH on this reaction showed that the immediate formation of the insoluble precipitate took place over the entire range examined; viz., from pH 2 to 11. This is noteworthy because of the possible

\* This work has been supported by a grant from the John and Mary R. Markle Foundation.

conclusion that certain proteins not as markedly basic as salmine, the isoelectric point of which has been found at 12 (2), may form compounds with cephalin at a physiological pH.

With lecithin no such compound formation took place within the physiological range. In strongly alkaline buffers, however, at pH 10 and 11, there occurred the formation of insoluble compounds.

This significant difference in the behavior of cephalin and lecithin towards the protamine may be understood from a consideration of the polar characteristics of these phosphatides.



Lecithin, which contains the strong base choline, is an internally neutralized compound. In recent determinations (3) its isoelectric point was found at 6.7. Cephalin, on the other hand, which contains the weaker base ethanolamine, has markedly acidic properties. According to Grün and Limpächer (4) and Rudy and Page (5) cephalin under certain conditions is titrated as a monobasic acid towards phenolphthalein, whereas lecithin is neutral. In a more recent investigation Fischgold and Chain (6) come to the conclusion that at acid reaction both lecithin and cephalin are able to bind 1 equivalent of  $\text{H}^+$  ions, whereas at alkaline reaction only cephalin gives up 1 equivalent of  $\text{H}^+$  ions.

As to the chemical nature of the extremely stable products obtained by the reaction of cephalin and salmine, the most likely assumption is that of salt formation between the strongly basic protamine and the acidic phosphatide. On the basis of their phosphorus and nitrogen contents the substances consisted of about 80 per cent of cephalin and of 20 per cent of salmine.

The bearing of the experiments here discussed on the problem of lipoproteins is obvious. Although the existence of lipid-pro-

tein complexes<sup>1</sup> in nature has long been assumed, experimental work with regard to the nature of these complexes has been scanty. One group of workers chose the preparatory approach to the problem by attempting the synthesis of complexes between serum or egg albumin (8-11), zein (10), or caseinogen (12) and lecithin. That these attempts did not lead to stable compounds may, in the light of the results here reported, be partly due to the fact that lecithin, in contrast to cephalin, is not appropriate for model experiments of this nature. Another group of workers (13-15) investigated the influence of proteins on the flocculation of lecithin sols. In these studies cephalin does not seem to have been used either.

Because of the inconclusive results obtained by other workers, orienting experiments were carried out on the formation of compounds between highly purified samples of egg albumin and lecithin or cephalin at various pH levels. It was of great interest to find that also with egg albumin immediate compound formation appeared to occur only in the case of cephalin. The precipitation of cephalin complexes took place at pH 2, 3, and 4. The much narrower range within which cephalin-albumin complexes are formed, as compared with the formation of compounds with salmine, is in entire harmony with the position of the isoelectric points of the two proteins, egg albumin having its isoelectric point at 4.8, salmine at 12.<sup>2</sup>

Lipoproteins have an important function in many biological processes. The natural activator of blood coagulation, the thromboplastic factor, apparently is a complex between cephalin

<sup>1</sup> The indiscriminate use of the term lipoproteins (*e.g.* (7)) for complexes between proteins and phosphatides, sterols, glycerides, and fatty acids respectively is confusing. Besides, one ought to distinguish between compounds, as the phosphatides, containing functional groups which make possible a combination with proteins by primary valence forces, and the sterols, for example, which, if they combine with proteins at all, could only do so by an attraction due to secondary valence forces.

<sup>2</sup> It will be of interest to study the formation of compounds between cephalin and proteins having more basic isoelectric points than egg albumin, which ought to take place within the physiological pH range. In view of the importance of cephalin-protein complexes in blood coagulation it should be noted that the isolation of two basic fibrinogen fractions has been reported with isoelectric points at pH 8.5 and 12.4 respectively (16) (compare, however (17)).



and a probably specific protein (18). The importance of lipid-protein complexes in immunology (19-21) can only be mentioned here.

When the salt-like nature of lipoproteins is put forward as a possible formulation of these compounds, it should be kept in mind that the fact that lecithin and cephalin usually occur together by no means connotes a common physiological function of the two. It is entirely possible that cephalin will be found to be a structural component of the organism, whereas lecithin may be concerned with the intermediary fatty acid metabolism. It is hoped that the use of the radioactive phosphorus isotope  $^{32}_{15}\text{P}$  as a label will be of value for investigations of this type.

#### EXPERIMENTAL

##### *Material*

*Cephalin* — Two different samples of cephalin were used in these experiments. One was a freshly isolated preparation from beef brain, obtained by extraction of the acetone-dried organ with petroleum ether (b.p. 30-60°). This material<sup>3</sup> was freed of cerebrosides by repeatedly freezing its solutions in ether and petroleum ether, and separated from lecithin by numerous precipitations with alcohol from petroleum ether solution. The *cephalin*, Fraction C-1, formed an almost white powder. Analysis, found, C 61.2, H 9.4, P 3.5, N 1.7, amino N 1.4, P:N = 1:1.1, P:amino N = 1:0.9.

The other cephalin preparation used was an older sample that had been extracted from sheep brain by means of ether and stored under CO<sub>2</sub> for some time. In order to purify this material two methods were attempted which, since they may be of general interest, will be briefly described. One method involved the chromatographic adsorption of the cephalin to aluminum oxide (activated according to Brockmann). A solution of 1.12 gm. of cephalin in 50 cc. of a mixture of equal parts of petroleum ether (b.p. 30-60°) and ligroin (b.p. 70-90°) was slowly filtered through an adsorption column (150 × 15 mm.). The chromatogram then was developed with 70 cc. of the petroleum ether-ligroin

<sup>3</sup> We are indebted to Mr. H. D. Hoberman for this preparation.

mixture. The colorless filtrate was concentrated *in vacuo*, and the ether solution of the residue was centrifuged, concentrated, and precipitated with acetone. The *cephalin*, Fraction C-2, weighed 0.44 gm. and formed a white powder. Analysis, found, C 55.8, H 9.0, P 4.0, N 1.9, amino N 1.7, P:N = 1:1.

The second method consisted in the distribution of the material between two solvents; *viz.*, petroleum ether-ligroin and diacetone alcohol,  $\text{CH}_3\cdot\text{CO}\cdot\text{CH}_2\cdot\text{C}(\text{CH}_3)_2\text{OH}$ . While the dry solvents are perfectly miscible, the addition of 1 per cent of water to the diacetone alcohol is sufficient to produce a sharp separation into two layers. Of the crude cephalin, 10.0 gm. were dissolved in 100 cc. of petroleum ether-ligroin (1:1), 40 cc. of freshly distilled diacetone were added, followed, after being mixed, by 0.4 cc. of water. The lower layer removed a considerable amount of impurities. The process was repeated five times, whereupon the solution of the cephalin in petroleum ether-ligroin was slowly dropped into 300 cc. of chilled absolute alcohol. The *cephalin*, Fraction C-3, weighed 7.4 gm. and formed a faintly yellow powder. Analysis, found, C 54.3, H 8.6, P 4.2, N 1.8, amino N 1.6, P:N = 1:0.94.

*Lecithin*—The lecithin used was freshly prepared from beef brain, as described above for the cephalin Fraction C-1. For purification it was converted into the cadmium chloride double salt. This salt was washed with ether, recrystallized from ethyl acetate, and decomposed by passing dry gaseous  $\text{NH}_3$  through its chilled solution in chloroform. After removal of the precipitate the chloroform solution was concentrated *in vacuo* and the *lecithin* was precipitated with acetone. It formed a slightly yellow plastic mass. Analysis, found; P 3.9, N 1.9, P:N = 1:1.1.

*Sphingomyelin*—In a few experiments pure sphingomyelin with a P:N ratio of 1:2 was used, which had been isolated from the spleen of a case of Niemann-Pick's disease.<sup>4</sup>

*Salmine Sulfate*—The preparation used was placed at our disposal by E. R. Squibb and Sons, New Brunswick, New Jersey. After purification by precipitation with alcohol from an aqueous solution it formed a white powder. Analysis, found, C 38.1, H 6.5, N 17.9, amino N 10.1, S 6.5.

<sup>4</sup> Chargaff, E., unpublished data.

*Compounds between Cephalin and Salmine*

The aqueous emulsions of the phosphatides used were obtained by treating the material in a mortar with a small amount of water, until a homogeneous emulsion was obtained, followed by gradual dilution with more water and filtration through a thick layer of cotton. In this manner very stable emulsions could be obtained.

To an emulsion of 3.07 gm. of cephalin, Fraction C-1, in 125 cc. of water a solution of 1.0 gm. of salmine sulfate in 50 cc. of water was added. A voluminous white precipitate separated immediately, which was filtered off and repeatedly washed with water and acetone. The product, which weighed 2.86 gm., formed an almost white powder which was soluble in moist ether, chloroform, and warm ethyl acetate and glacial acetic acid. On contact with water, in which it is insoluble, it formed a rubber-like elastic mass. This substance, like all the other protamine-cephalin complexes here described, had no true melting point. When slowly heated in a capillary tube, it softened at about  $140^{\circ}$  and gradually decomposed to form a dark brown, viscous oil. None of the compounds described contained sulfur. Analysis, found, P 2.9, N 4.7, P:N = 1:3.5. The cephalin content of this preparation may be computed to be 84 per cent on the basis of the phosphorus value and 85.2 per cent on the basis of the nitrogen value.

A solution of 1.0 gm. of this substance in ether was repeatedly shaken with dilute sulfuric acid. The ethereal solution was washed with water, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated to a small volume. After precipitation with acetone 0.72 gm. of a white powder was obtained which had the same properties as the original product. Analysis, found, P 3.1, N 5.1, P:N = 1:3.6.

From a filtered solution of 500 mg. of the original product in 20 cc. of warm chloroform 440 mg. of a practically unchanged substance were obtained by precipitation with acetone. Analysis, found, P 3.1, N 5.0, P:N = 1:3.5.

The cephalin-salmine complex can also be recovered unchanged from hot ethyl acetate. A filtered solution of 300 mg. of the substance in 15 cc. of hot ethyl acetate on cooling yielded 240 mg. of a slightly yellow amorphous powder. Analysis, found, P 3.0, N 4.6, P:N = 1:3.4. This complex contained 85 per cent of cephalin according to both the nitrogen and phosphorus values.

In order to ascertain whether the relative proportion of cephalin and salmine materially affected the composition of the final product, in another experiment the complex was prepared with an emulsion of 1.0 gm. of cephalin, Fraction C-1, and 1.0 gm. of salmine in a total volume of 100 cc. of water. The product obtained weighed 0.95 gm. Analysis, found, P 2.8, N 5.9, P:N = 1:4.7. This complex contained 80.5 per cent of cephalin on the basis of its phosphorus value and 79.1 per cent according to the nitrogen value.

From another cephalin preparation, Fraction C-3, a similar substance was obtained. From 125 cc. of a 1.3 per cent cephalin emulsion and 25 cc. of a 2.2 per cent salmine solution, 1.61 gm. of the complex were prepared. Analysis, found, P 2.9, N 6.5, P:N = 1:5. The composition of this compound was likewise not changed by treatment with acids or recrystallization.

The experiments here described tend to demonstrate the remarkable stability of the complexes between cephalin and salmine.

*Lecithin or Sphingomyelin and Salmine*—A number of experiments with lecithin and salmine in varying proportions, as described above for cephalin, did not lead to any definite compounds. Several hours after the addition of the protamine solution to the lecithin emulsion a slight flocculation had taken place. The fine floccules were centrifuged off. After repeated washing with water, in which they could be easily emulsified, and treatment with acetone, unchanged lecithin was recovered.

Because of the limited amount of pure sphingomyelin at our disposal, only a few orienting experiments were carried out. These showed, however, that sphingomyelin was not precipitated by the addition of salmine.

*Influence of pH on Precipitation of Phosphatide-Salmine Complexes*—In order to study the formation of the compounds between phosphatides and salmine at different hydrogen ion concentrations, a series of experiments in buffer solutions was carried out. Between pH 1.92 and 5.96 citrate buffers were used; between pH 6.97 and 9.18 phosphate buffers; for pH 10.1 and 11 ammonia-ammonium chloride buffers were employed. The results are reproduced in Table I. As control, the behavior of lecithin emulsions at different pH levels without the addition of salmine was examined. The results, reproduced in Table II, are

TABLE I

*Reaction between Phosphatides and Salmine at Various pH Levels*

Each tube contained 10 mg. of the phosphatide emulsified in 0.5 cc. of water, 0.5 cc. of the buffer, and 5 mg. of salmine dissolved in 0.5 cc. of water.

pH	Cephalin, immediately after addition of salmine	Lecithin	
		Immediately after addition of salmine	80 min. after addition of salmine
1.9	+	—	—
3.0	+	—	×
3.9	+	—	×
5.0	+	—	×
6.0	+	—	×
7.0	+	—	×
8.0	+	—	×
9.2	+	—	×
10.1	+	+	
11.0	+	+	

+ = heavy precipitate which could not be reemulsified. × fine flocculation, easily reemulsified. — = no flocculation.

TABLE II

*Stability of Lecithin Emulsions at Various pH Levels*

Each tube contained 10 mg. of lecithin emulsified in 1 cc. of water and 0.5 cc. of the buffer.

pH	Lecithin	
	After 40 min.	After 80 min.
1.9	—	—
3.0	×	×
3.9	×	×
5.0	—	×
6.0	—	×
7.0	—	—
8.0	—	—
9.2	—	—
10.1	—	—
11.0	—	—

× = fine flocculation, easily reemulsified. — = no flocculation.

in general agreement with the findings of Remesow (22). In the experiments recorded in Tables I and II no additional changes were observed in repeated readings during a period of 18 hours. Emulsions of cephalin alone were stable at all pH levels studied.

These experiments showed clearly that cephalin reacted with salmine within the entire range between pH 2 and 11, lecithin only at pH 10 and 11. At lower pH levels, the addition of salmine to an emulsion of lecithin seems merely to decrease the

TABLE III

*Reaction between Phosphatides and Egg Albumin at Various pH Levels*

Each tube contained 10 mg. of the phosphatide emulsified in 0.5 cc. of water, 0.5 cc. of the buffer, and 5.3 mg. of egg albumin dissolved in 0.5 cc. of water.

pH	Cephalin, immediately after addition of egg albumin	Lecithin	
		Immediately after addition of egg albumin	80 min. after addition of egg albumin
1.9	+	—	—
3.0	+	—	×
3.9	+	—	×
5.0	—	—	×
6.0	—	—	×
7.0	—	—	—
8.0	—	—	—
9.2	—	—	—
10.1	—	—	—
11.0	—	—	—

+ = heavy precipitate which could not be reemulsified. × fine flocculation, easily reemulsified. — = no flocculation.

stability of the emulsion. It should perhaps be mentioned that at the concentrations studied salmine itself did not show any spontaneous precipitation.

*Precipitation of Phosphatide-Egg Albumin Complexes*—In these experiments highly purified egg albumin was used. This material which had been twelve times recrystallized was kindly placed at our disposal by Dr. M. Heidelberger. The experiments carried out were similar to those with salmine described in the preceding paragraphs. From the results summarized in Table III it will be seen that cephalin immediately formed characteristic

precipitates with egg albumin at pH 2, 3, and 4, whereas with lecithin the same type of slow flocculation of the emulsions occurred as in the control experiments reproduced in Table II.

The author is indebted to E. R. Squibb and Sons, New Brunswick, New Jersey, for the sample of salmine sulfate. He wishes to thank Mr. W. Saschek for numerous microanalyses, and Mr. Bernard Kress for general assistance.

#### SUMMARY

The preparation of compounds between cephalin and salmine and the properties of these substances are described. The significance of the findings with regard to the problem of lipoproteins is discussed.

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# STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

## VII. PROTAMINES AND BLOOD CLOTTING\*

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An investigation of the rôle of protamines in blood coagulation must start from the following facts. (a) Protamines act as anti-coagulants both *in vivo* (1) and *in vitro* (2). (b) Protamines inhibit the anticoagulant effect of heparin both in the living organism and in plasma (3). To these observations a third must now be added. Cephalin is precipitated from its emulsions in water by the protamine salmine which forms with it an insoluble complex (4). Protamines are of interest in an analysis of the blood clotting mechanism, as they combine with two important agents which, although not antagonists, have opposite effects on clotting; *viz.*, with heparin which inhibits, and with cephalin which activates the coagulation of blood and plasma.

The present paper has the double purpose of supplementing a previous communication from this laboratory (3) by furnishing a brief description of the compound between heparin and salmine, and of analyzing the effects of protamines on clotting inhibitors and activators.

### EXPERIMENTAL

*Compound between Heparin and Salmine*—In these experiments a heparin preparation from beef lungs (5) was used which had an anticoagulant potency of 530 inhibitor units per mg. (6). A neutral solution of 1.01 gm. of this material in 50 cc. of water was made slightly acid with acetic acid and filtered. On addition of 0.37 gm. of salmine sulfate in 30 cc. of water immediate precipita-

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tion took place. After addition of 1.5 cc. of glacial acetic acid the mixture was chilled for several hours. The precipitate which was removed by centrifugation formed a slightly yellow, elastic sheet. It was washed with 5 per cent acetic acid and subsequently triturated with absolute alcohol, when it disintegrated to a faintly yellow powder which after drying weighed 0.48 gm. Analysis, found, N 14.3, S (volatile) 6.7, ash 0.8, amino sugar 11.7 (calculated as glucosamine).<sup>1</sup>

Heparin which probably is a high molecular sulfuric acid ester (8) is usually found to contain a large amount, about 35 per cent, of inorganic sulfates as ash. The fact that our product was practically free of ash and contained the total sulfur in volatile form shows that the strongly basic protamine had replaced the cations of heparin to form a salt between salmine and heparin.

As was to be expected from our previous experiments (3), a fine suspension of the heparin-salmine salt in physiological saline did not show any effect on clotting, when examined according to the method employed in this laboratory (6).

The complex between heparin and salmine here described is to a certain degree reminiscent of the compounds of clupein with various acids described by Felix and Mager (9) and of the protein complexes of chondroitinsulfuric acid prepared by Meyer, Palmer, and Smyth (10).

The protamine-heparin complex was found remarkably stable. Repeated treatment with 10 per cent  $\text{CaCl}_2$  solution (11), for instance, did not set free any heparin. Even by extended tryptic digestion only little heparin, although of higher purity, was liberated. A suspension of 390 mg. of the salt in 15 cc. of 0.5 N ammonia-ammonium chloride buffer of pH 9.2 was incubated with 50 mg. of trypsin (Fairchild) at 38° for 48 hours. During this period the mixture did not change its appearance appreciably. The undissolved material was centrifuged off and washed with 1 N ammonia. The fraction that had gone into solution was precipitated from the combined supernatant liquids with acetone, centrifuged off, washed with acetone and ether, and dried. It weighed 53.5 mg. and formed an almost white powder. The

<sup>1</sup> The author wishes to thank Dr. K. Meyer of this School for the amino sugar determination which was carried out according to the method of Palmer, Smyth, and Meyer (7).

recovered *heparin* showed a considerably higher anticoagulant potency (2500 inhibitor units per mg.) than the original product.

In order to arrive at a better characterization of this heparin fraction it was converted into the barium salt according to the method of Charles and Scott (12). The product obtained consisted of microscopic needles, weighing 31.9 mg., but was not identical with the crystalline barium salt of heparin described by these authors. Analysis, found, C 26.6, H 5.4, N 3.1, S 8.6, ash ( $\text{BaSO}_4$ ) 21.4, Ba 12.6. It can be seen that there was almost twice as much volatile S. as was contained in the ash. The anticoagulant activity of this fraction was 1600 inhibitor units per mg.

A study of the influence of the pH on the precipitation of the heparin-salmine compound, as described in the preceding paper (4), showed compound formation to take place over the entire range examined between pH 2 and 11.

*Salmine and Anticoagulants*—The anticoagulants examined fall into two distinct groups with regard to their behavior towards salmine. The following substances were practically completely inactivated by the protamine: heparin (3), cellulose sulfuric acid, polyvinyl sulfuric acid, the lipid inhibitors of clotting recently isolated from brain and blood cells (13), and the synthetic cerebrin sulfuric and kersin sulfuric acids (14). It will be seen that all the synthetic substances are, and the natural anticoagulants may be assumed to be, strong acids which in all likelihood combine with the basic protamine to form insoluble salts. It is remarkable that the combination of the salmine with heparin can be effected during a considerable period after the injection of the latter into the blood stream or the addition of it to plasma *in vitro*.

The anticoagulants not affected by protamines comprise sodium oxalate and sodium citrate.

The addition of salmine, which is itself an anticoagulant, to plasma containing heparin brings about a considerable decrease in the coagulation time of the plasma. It, therefore, was thought possible to ascertain by means of the protamine reaction whether normal plasma contained an anticoagulant of the heparin type. In these experiments very small amounts of salmine (0.06 to 8.5 micrograms) were added to 0.1 cc. of unactivated chicken plasma (6). There was, however, no decrease in the clotting time, as might have been expected if small amounts of heparin had been

bound by the protamine. One may conclude that heparin, at least in an uncombined state, does not occur in chicken plasma.

*Salmine and Cephalin*—Cephalins, or possibly certain representatives of the cephalin group, are known to be potent activators of blood clotting (compare Figs. 1 and 2 (15)). It is assumed that the thromboplastic factor contained in most tissues is a complex between cephalin and a protein.

In the preceding paper the preparation of cephalin-salmine complexes was described (4). The addition of a suspension of this material to unactivated chicken plasma had no effect whatever on the plasma clotting time. This was in sharp contrast with the high activating potency of the uncombined cephalin. These

TABLE I  
*Effect of Salmine on Clotting of Cephalin-Containing Chicken Plasma*

Cephalin in 0.1 cc. chicken plasma	Clotting time	
	Experiment A	Experiment B
mg.	min.	min.
0.032	5	25
0.016	10	30
0.008	15	30
0.004	25	35
0.002	30	40
0	30	45

In Experiment A the chicken plasma contained only cephalin; in Experiment B 0.005 mg. of salmine had been added to each sample of cephalin-containing plasma.

experiments showed that under proper conditions cephalin and salmine were able to counteract each other's action: the activator cephalin reacted with the inhibitor salmine.

The antagonistic effect of salmine on the activation of plasma clotting by cephalin can also be demonstrated, although not as clearly, by the addition of small amounts of salmine to cephalin-containing chicken plasma. A typical experiment is reproduced in Table I.

#### DISCUSSION

The ability of protamines to destroy the inhibiting effect of anticoagulants may find various applications which have been

discussed in a previous publication from this laboratory (3). In the experiments cited above the protamine reaction failed to establish the presence of small amounts of heparin in normal chicken plasma. The presence of heparin or a similar substance in the blood of dogs during anaphylaxis and in peptone shock has recently been demonstrated by means of the protamine reaction (16).

In view of the results reported here a new explanation for the anticoagulant effect of protamines may be put forward. The anticoagulants can be grouped into (a) substances reacting with the calcium ion necessary for clotting (sodium citrate or oxalate), (b) substances reacting with the prothrombin or thrombin (or, perhaps, with the fibrinogen) either by blocking certain groups that are necessary for the conversion of fibrinogen into fibrin, or by offering a substitute substrate to the enzyme, thereby protecting the fibrinogen (heparin, synthetic anticoagulants, hirudin), and (c) substances reacting with the cephalin or the thromboplastic factor. No anticoagulant of the third group has as yet been satisfactorily demonstrated. From the experiments reported here it can be concluded that the action of protamines in blood clotting is at least partly due to the inhibition of the clotting activator.

The author is indebted to Hoffmann-La Roche, Inc., Nutley, New Jersey, for some of the heparin used in the experiments, and to E. R. Squibb and Sons, New Brunswick, New Jersey, for a sample of salmine sulfate. Thanks are due to Miss Hildegard Menzel and Mr. Bernard Kress for assistance in the course of these experiments.

#### SUMMARY

The properties of a heparin-salmine complex are described. It is shown that the complexes between salmine and heparin or cephalin are both inactive in blood clotting. The effect of protamines on plasma containing inhibitors or activators of blood clotting is discussed. The anticoagulant effect of salmine is explained on the ground that it combines with the natural activator of coagulation.

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## STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

### VIII. ISOLATION OF A LIPID INHIBITOR OF BLOOD CLOTTING FROM THE SPLEEN IN A CASE OF NIEMANN-PICK'S DISEASE\*

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In recent communications from this laboratory (1, 2) has been reported the isolation from brain, spinal cord, and blood cells, of lipid fractions which acted as inhibitors of blood clotting. It was demonstrated that these compounds were associated with the sphingomyelin fraction and that substances of similar activity could be synthesized by esterification of cerebrin and kersin with sulfuric acid (3).

In the course of a study of the chemical composition of the spleen in a case of Niemann-Pick's disease, which will be presented in the near future, particular attention was devoted to the question whether in this case also the sphingomyelin fraction contained a lipid inhibitor. If one assumes that in primary disturbances of metabolism, metabolic occurrences which in the normal organism take place to a slight degree only or as intermediary stages, are enormously magnified, the presence of the lipid inhibitor in a case of lipidosis, as Niemann-Pick's disease, will tend to emphasize its natural rôle as a companion of sphingomyelin. After inconclusive analyses had been reported by various workers, Klenk (4) succeeded in showing that the phosphatide deposited in large amounts in the spleen, liver, and brain in Niemann-Pick's disease, consisted mainly of sphingomyelin.

It, therefore, was of interest to find that the sphingomyelin fraction extracted by chloroform-methyl alcohol from the spleen

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in a case of this disease contained a potent anticoagulant which could be separated from sphingomyelin by precipitation from ligroin solution by absolute alcohol. The most active inhibitor fraction obtained, which had a higher potency than the fractions described previously (2), contained in addition to nitrogen and phosphorus an appreciable amount (2.7 per cent) of sulfur. This is in harmony with the view stated before (2, 3) that the lipid inhibitors, like many other anticoagulants (5), are sulfuric acid esters.

#### EXPERIMENTAL

*Extraction of Spleen*—The organ used had been removed by operation from an 8 month-old male child suffering from Niemann-Pick's disease. The spleen weighed 180 gm. The extraction of the lipids was in the main carried out according to Klenk (4). In the present paper only the sphingomyelin fraction will be discussed, the presentation of the other results being reserved for a subsequent publication.

The part of the spleen placed at our disposal (105 gm.) was twice extracted in the Soxhlet apparatus with acetone (for 14 and 8 hours), then once with ether for 18 hours, and once for 12 hours with methyl alcohol-chloroform (3:1). All the solvents were freshly purified. The acetone extract on cooling deposited 3.6 gm. of a mixture of sphingomyelin, monoaminophosphatides, and cholesterol, from which by the usual purification and recrystallization from ethyl acetate 1.04 gm. of pure *sphingomyelin* were obtained.<sup>1</sup> This fraction had no anticoagulant activity.

The main portion of crude *sphingomyelin* (5.5 gm.) was obtained from the chloroform-methyl alcohol extract by concentration and precipitation with acetone. This fraction showed inhibiting activity.

*Fractionation of Crude Sphingomyelin*—The fraction was almost completely insoluble in cold ether. After treatment of the total with ether 5.4 gm. of a light brown powder were obtained. The

<sup>1</sup> It should be pointed out that, in view of previous experiences concerning the anticoagulant effect of cadmium salts (2), the use of cadmium derivatives for the purification of the substances discussed in this paper had to be avoided.

anticoagulant activity of this material, *Fraction 1*, is shown in Table I.

Fraction 1 was treated with 40 cc. of warm glacial acetic acid in the usual manner (2). The absence of cerebrosides was demonstrated by the almost complete solubility of the material in acetic acid. The substance recovered from the concentrated

TABLE I  
*Inhibiting Activities of Fractions from Sphingomyelin*

Fraction No.	Amount in 0.1 cc. plasma	Clotting time	
		Activated chicken plasma	Chicken plasma
	mg.	min.	min.
1	0	6	
	0.04	8	
	0.08	8	
	0.17	10	
	0.34	20	
	0.68	30	
3	0	6	
	0.015	6	
	0.03	12	
	0.06	18	
	0.12	27	
	0.24	36	
4	0.48	55	
	0	16	50
	0.03	21	65
	0.07	28	125
	0.15	45	335
	0.30	105	>365
	0.60	>240	>365

solution by precipitation with acetone, *Fraction 2*, weighed 5.13 gm. and showed the same activity as Fraction 1.

To a solution of 5.08 gm. of Fraction 2 in 35 cc. of ligroin-absolute alcohol (5:1) 70 cc. of absolute alcohol were added. The mixture was chilled and filtered; the precipitate was washed with alcohol and dried. The product, *Fraction 3*, 0.79 gm. of a light brown powder, possessed increased anticoagulant potency, as



shown in Table I. The pure sphingomyelin recovered from the alcohol solution was inactive.

Fraction 3 (0.74 gm.) was treated with 25 cc. of a warm mixture of 5 parts of ligroin and 1 part of absolute alcohol. The insoluble fraction was again treated with the same solvent mixture, separated by centrifugation, and dried. The resulting material, *Fraction 4*, weighed 0.14 gm. and formed a brown powder. Analysis, found, N 7.5, P 3.3, S 2.7. This fraction was a very potent anticoagulant, as shown in Table I.

The author is indebted to the Medical Staff of Babies Hospital for the spleen used in these experiments. Thanks are due to Mr. W. Saschek for the microanalyses and to Miss Hildegard Menzel for technical assistance.

#### SUMMARY

The isolation and properties of a lipid fraction from the spleen of a case of Niemann-Pick's disease are described, which inhibits the clotting of blood.

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## PROPERTIES OF THE ANTIHEMORRHAGIC VITAMIN (VITAMIN K)

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The effect of certain reagents and treatments on the antihemorrhagic vitamin (K) has been discussed in former papers (1, 2). This work has been repeated and extended. In addition, several attempts have been made to form derivatives of vitamin K that would provide a way for further purification. The present physical methods for purification of the vitamin (2) have reached about the limit of their effectiveness. It is hardly necessary to state that the final products of such methods may easily be a mixture of constant composition rather than the pure vitamin. Hence, further means of purifying the vitamin must be discovered if the vitamin is to be isolated in what may be considered a condition of purity.

### *Methods and Results*

Biological assays were conducted in accordance with a procedure already described (3). For the most part chicks were fed the basal diet for the 1st week, then fed supplemented diets up to an age of 2 weeks. Suitable positive control groups were included in each assay. These groups received diets supplemented with known quantities of a reference standard consisting of a crude hexane extract of dried alfalfa, preserved for such purpose. Negative control groups receiving only the basal diet were also maintained throughout an assay. Five to ten chicks were placed in each test group.

The concentrate used was that obtained by distillation under greatly reduced pressure (2). The quantity of this concentrate per kilo of diet required to bring the average blood clotting time

of chicks to a value below 5 minutes was determined in advance. Then similar quantities of the vitamin concentrate were treated in various ways and fed to chicks in a comparable manner. When polar solvents were used in the chemical treatments, the material fed was the hexane extract of the reaction mixture, this extract having been washed with water until all water-soluble substances had been removed. When non-polar solvents were used, the reagents were washed from the final product by suitable aqueous solutions, the non-polar solvent evaporated *in vacuo* at room temperature, and the oily residue taken up in hexane.

The former experiments with phenyl isocyanate and 3,5-dinitrobenzoyl chloride (1) were repeated and confirmed. Neither of these reagents for an alcoholic hydroxyl group appeared to form a derivative or to diminish the activity of the concentrate. Treatment with benzoyl chloride seemed to cause some loss of activity. The loss, however, was traced to the pyridine used as solvent in this particular case and, after redistillation of the pyridine, the use of benzoyl chloride had no detectable effect on the activity of the concentrate. A quantity of the concentrate was heated with acetic anhydride at 100° for 1 hour. Hexane was then added and the hexane solution washed several times with water. The material in the hexane extract was fully as active as the original concentrate.

An attempt was made to prepare the allophanate derivative of the vitamin according to the procedure of Windaus (4). A benzene solution of the concentrate was saturated with cyanic acid gas and allowed to stand at 0° for 2 weeks. The assay of this benzene solution demonstrated no loss in potency.

The former experiments with bromine and iodine in carbon tetrachloride (1) were repeated and the same results obtained. 10 minutes contact with bromine in carbon tetrachloride in the dark at room temperature completely destroyed the potency of the concentrate, while a similar treatment with iodine had no effect. Additional bromination experiments were made in which an attempt was made to restore the potency of the bromination product by regenerating any double bonds which had been saturated by the bromine. The bromination was carried out in benzene solution in the dark. The regeneration of the double

bonds was attempted with zinc and glacial acetic acid in one experiment, and with potassium iodide in absolute ethyl alcohol in another experiment. In both cases there was no activity in the final product. Treatment of the concentrate with the mild brominating agent, 3,5-dibromopyridine, in carbon tetrachloride in the dark for 48 hours had no effect on the potency.

When the concentrate was allowed to react with a dilute solution of hydrogen iodide in glacial acetic acid in the dark for 24 hours, the activity was completely destroyed.

In general, oxidizing agents had a destructive action on the vitamin. A few drops of concentrated nitric acid were added to a solution of the concentrate in glacial acetic acid. The nitric and acetic acids were removed by adding hexane and washing the hexane extract with water several times. If the concentrate was allowed to remain in contact with the nitric acid for 4 hours at room temperature, there was no loss in potency; 24 hours at room temperature resulted in a small loss, while half an hour's contact at 100° completely destroyed the potency of the vitamin. A distinctly positive test for an aromatic nitration product was obtained when the oily residue from the evaporation of the final hexane extract was dissolved in absolute ethyl alcohol, and a solution of potassium hydroxide in ethyl alcohol added.

Treatment of the concentrate with sodium dichromate in glacial acetic acid solution for 24 hours at room temperature resulted in a partial loss in activity; the same treatment for half an hour at 100° gave complete destruction. When the concentrate was allowed to remain in contact with ferric chloride in glacial acetic acid for 24 hours, there was partial loss in potency.

Reducing agents, acting as such, had little or no effect upon the vitamin. Treatment of the concentrate with stannous chloride in glacial acetic acid at 100° for half an hour had no effect on the potency; nor did a similar treatment with metallic iron have any destructive results.

A 24 hour treatment with sodium sulfite in glacial acetic acid partially destroyed the activity. The potency of the concentrate was completely destroyed by a 24 hour exposure to a saturated solution of hydrogen sulfide in glacial acetic acid.

The effect of hydrogen cyanide on the activity was noted by

heating the concentrate at 100° for half an hour in a solution of glacial acetic acid to which a small amount of sodium cyanide had been added. There was no resulting loss in potency.

Exposure of the concentrate in a hexane solution to concentrated sulfuric acid for 24 hours at room temperature gave complete loss of activity. Phosphorus pentoxide also caused considerable loss of activity when kept in contact with a hexane solution of the concentrate at room temperature for a 24 hour period.

In further investigations on the effect of acids, a crude hexane extract of alfalfa of known vitamin K potency was evaporated *in vacuo* on a water bath, dissolved in a dilute solution of hydrochloric acid in methyl alcohol, and refluxed on a water bath for 2 hours. No appreciable amount of the original activity was left after this treatment. However, treatment of the more refined concentrate with a very dilute solution of hydrochloric acid in ethyl alcohol for 15 minutes at refluxing temperature had no measurable effect on the potency.

Two tests were made of the action of anhydrous aluminum chloride on a hexane solution of the concentrate. In both cases, one for 1 hour at room temperature and the other for half an hour at 100°, there was a large or complete loss of activity.

The concentrate was dissolved in a methyl alcohol solution containing 10 per cent of purified formaldehyde. After the solution had been refluxed for 5 minutes, it was allowed to stand at 0° for 2 days and then assayed. This treatment caused no loss in activity. Heating the concentrate in a small volume of redistilled furfural at 100° for half an hour likewise had no effect.

Refluxing the concentrate in carbon disulfide and in a solution of sulfur in carbon disulfide produced no destruction of the activity.

The apparent molecular weight of the vitamin was determined by the camphor method of Rast (5). 40° was used as the molal freezing point depression of camphor. The material used in the determination was a relatively very pure product obtained by chilling the vitamin out of a methyl alcohol solution as a solid by the use of solid carbon dioxide, centrifuging, and discarding the mother liquor. This chilling out process was carried out three successive times. Two determinations, one in which 5.4 mg. of the vitamin concentrate were dissolved in 26.2 mg. of camphor, and the other in which 5.6 mg. of the vitamin concentrate were

dissolved in 100 mg. of camphor, both gave values of the molecular weight of approximately 525, which, from a consideration of the inherent limitations of the method, should be accurate to within 5 per cent. Since the degree of purity of the concentrate is not known, the apparent molecular weight can be taken only as a preliminary estimation.

#### DISCUSSION

The inability of reagents which are known to couple with alcoholic hydroxyl groups to alter the activity, solubility, or other characteristics of the vitamin seems to point to the non-existence of such alcoholic groups in the vitamin molecule. It is conceivable that esters of vitamin K with the natural acetic and benzoic acids might be fully active, if such were formed. However, the fact that 3,5-dinitrobenzoyl chloride, phenyl isocyanate, and cyanic acid failed to affect the activity of the concentrate indicates strongly that the vitamin has no alcoholic group. The similar failure of hydrogen cyanide indicates the absence of carbonyl groups.

Irreversible loss of activity due to bromination suggests more than an ordinary saturation of aliphatic double or triple bonds. In addition to saturating any such linkages present, the bromine may well be substituting in an aromatic portion of the molecule, the existence of which is indicated by the positive aromatic nitration product test and the destructive action of aluminum chloride.

The destructive action of oxidizing agents is to be expected, since any organic substance of such large molecular weight must have many vulnerable points of attack.

Reducing agents had slight effect except in cases where the reagent also had the ability to add across a double bond; that is, with hydrogen iodide and hydrogen sulfide.

The action of acids, as such, appears to result in varying degrees of destruction. Whether this is due to a catalytic effect of the hydrogen ion or a direct reaction between the acid and vitamin molecule is an open question.

Failure of the two aldehydes, formaldehyde and furfural, both exceptionally good condensing agents, to effect any change in the activity would seem to indicate the absence of phenolic groups or similarly susceptible groups in the vitamin molecule.

The properties of the vitamin are consistent with those of a complex, unsaturated hydrocarbon.

#### SUMMARY

Further studies on the effect of various chemical reagents on the antihemorrhagic vitamin have been made. The activity of the vitamin was destroyed by oxidizing agents, strong acids, aluminum chloride, and several reagents which have the ability to add across an ethylenic linkage, but was not appreciably affected by reducing agents or by reagents which react with alcoholic, carbonyl, or phenolic groups.

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## EVIDENCE OF A NEW GROWTH FACTOR REQUIRED BY CHICKS

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During experiments on the formulation of a diet for riboflavin assay with chicks, it was observed that middlings and yeast had a growth-promoting effect which could not be accounted for by their filtrate factor or riboflavin content. Almquist (unpublished data) has also observed that a diet of polished rice, fish meal, dried yeast, salt, and cod liver oil gave better growth than a similar diet in which the yeast had been replaced by concentrates of riboflavin, filtrate factor, and thiamine.

The experiments reported here were planned to study this growth-stimulating factor (or factors) in yeast and middlings. The results indicate that the growth factor involved is distinct from any of the nutritional factors thus far described as being required by the chick. Studies were also made on the distribution and chemical properties of the factor.

### *Method and Materials*

In all the experiments white Leghorn chicks were used, being maintained in electrically heated batteries and given feed and water *ad libitum*. Except where otherwise indicated they were used in groups of ten to twelve. In the first three experiments, day-old chicks were started on the experimental diets and continued for 42 days. In later experiments, as will be indicated, the chicks were placed on a depletion diet for 14 days, after which they were given the assay diets for an additional 14 days.

The five diets used are described in detail in Table I. Diets 1, 2, and 3 differ essentially in the cereal bases employed. Diet 1A is the same as Diet 1 with the exception that the polished



rice was increased from 50 to 58.5 per cent, the starch being omitted. The washed fish meal in the diets containing polished rice was increased from 20 to 24 per cent to compensate for the lower protein content of the polished rice.

The washed fish meal was prepared by washing a commercial sardine meal ten times with about 15 volumes of hot water (60-70°) and then drying in a vacuum oven. The whey adsorbate was prepared by adding 1 per cent of fullers' earth to liquid whey, stirring, allowing to settle, washing twice with water, and drying

TABLE I  
*Composition of Diets*

The figures are given in per cent, except for thiamine which represented 0.2 mg. per 100 gm. of diet.

Diet No.....	1	2	3	1A	38
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Polished rice.....	50			58.5	61.5
Ground yellow corn.....		55	35		
Wheat middlings.....			20		
Water-washed fish meal.....	24	20	20	24	24
Soy bean oil.....	3	3	3	3	3
High potency sardine oil (3000 units vitamin A and 400 units vitamin D per gm.).....	0.5	0.5	0.5	0.5	0.5
Salt mixture (see text).....	3	3	3	3	3
Rice bran filtrate.....	5	5	5	5	
Whey filtrate factor concentrate.....					2
" adsorbate.....	5	5	5	5	5
Ground limestone.....	1	1	1	1	1
Thiamine.....	0.2			0.2	0.2
Starch.....	8.5	7.5	7.5		

in a current of warm air. 5 per cent of this whey adsorbate furnished more than the required amount of riboflavin as determined by a separate assay on a low riboflavin diet. The rice bran filtrate<sup>1</sup> was prepared by treating a rice bran extract with fullers' earth. This rice bran filtrate had been assayed on a heated diet by the method of Lepkovsky and Jukes (1) and found to furnish adequate filtrate factor at a level of 3 per cent in the diet.

<sup>1</sup> Supplied through the courtesy of Vitab Products, Inc., Emeryville, California.

The filtrate factor employed in the basal ration Diet 38 was prepared from whey. Dried whey was extracted with hot methanol in a continuous extractor, the methanol removed from the extract under reduced pressure, water added, and the riboflavin removed by treatment with fullers' earth. This concentrate was assayed for the filtrate factor and was fed at a level assuring adequate amounts of this factor. The salt mixture employed had the following composition: 34 per cent NaCl, 31 per cent  $K_2H \cdot PO_4$ , 14 per cent  $MgSO_4 \cdot 7H_2O$ , 2.5 per cent  $FeSO_4 \cdot 7H_2O$ , 0.2 per cent KI, 0.3 per cent  $CuSO_4 \cdot 5H_2O$ , 0.2 per cent  $ZnCl_2$ , 0.5 per cent  $Al_2(SO_4)_3$ , 0.8 per cent  $MnSO_4 \cdot 2H_2O$ , and 16.5 per cent  $Na_2SiO_3 \cdot 9H_2O$ .

#### EXPERIMENTAL

*Demonstration of a Growth Factor in Yeast, Middlings, and Corn*—Diets 1, 2, and 3 were each fed with and without a supplement of pure dried brewers' yeast. The growth results, presented in Fig. 1, show that the replacement of 20 per cent of the corn in Diet 2 by 20 per cent of middlings increased the growth made in 6 weeks from 302 to 419 gm. The addition of yeast to the diet containing corn and middlings further increased the growth from 419 to 535 gm. The addition of yeast to the ration containing only corn as the cereal base produced the same growth as that attained on yeast, corn, and middlings, showing that the yeast also contains the factor furnished by the middlings. In the absence of added yeast the polished rice diet supported the least growth. The addition of yeast to this diet resulted in almost the same growth rate attained on Diets 2 and 3 when supplemented with yeast. On the basis of this experiment, it would seem that of the three cereals used, rice was the poorest, corn the next, and middlings the richest in the unknown growth factor.

*Distribution of Growth Factor*—The distribution of the growth factor in pure dried brewers' yeast, wheat bran, rice bran, sun-dried alfalfa leaf meal, and molasses was determined by adding these supplements to Diet 1A. This ration was used because it had been found that polished rice was low in the unknown growth factor. The supplements used replaced an equal amount of polished rice.

The growth results obtained are presented in Table II. They

show that 3 per cent of dried yeast was adequate for growth. Wheat bran and sun-dried alfalfa leaf meal were adequate at levels of 10 per cent, while rice bran at a 10 per cent level was

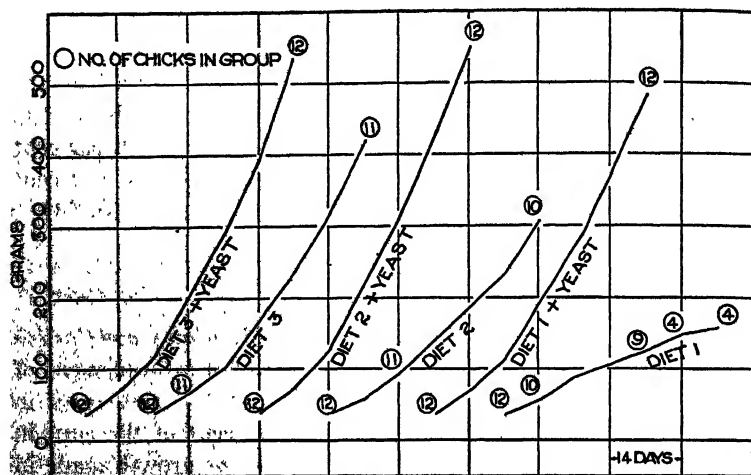


FIG. 1. The growth-stimulating effect of yeast, middlings, and corn. Yeast was fed at a level of 7.5 per cent.

TABLE II

*Growth-Promoting Properties of Various Substances Added to Polished Rice Diet (1A)*

Supplement	Weight, 42 days	Mortality, 42 days
	gm.	per cent
Polished rice diet (1A).....	215	55
3% dried yeast.....	413	9
10% wheat bran.....	434	9
10% rice bran.....	426	9
10% sun-dried alfalfa leaf meal.....	354	0
5% dried molasses.....	433	0
	138	90

slightly inadequate. Dried molasses contained little or none of this factor. Other assays have also shown the factor to be present in a preparation of yeast grown in a whey medium to which had been added mineral and carbohydrate yeast nutrients. The

preparation was concentrated and dried in a Peebles spray drier at a low temperature. The product leaving the drier had a temperature of about 40°.

*Solubility in Methanol and Water, and Stability to Autoclaving*—To determine the solubility of the factor in methanol and water and its stability to autoclaving the following experiments were performed. A sample of hexane-extracted alfalfa was extracted for 24 hours with hot methanol, and another sample washed with five portions of water at 60–70°. The water extract was clarified by filtration. Hexane-extracted alfalfa was used because it was available and because it was thought that the removal of part of the fat-soluble material by hexane would facilitate water extraction. An extract was also prepared from dried brewers' yeast by extracting 1 kilo of yeast three times with 4000 ml. portions of 50 per cent (by volume) methanol. The extracts from the alfalfa and the yeast were concentrated under reduced pressure and the residues dried in a current of warm air. The stability of the factor was determined by autoclaving samples of dried brewers' yeast and hexane-extracted alfalfa for 5 hours at 120°. Assay Diet 1A was used, the experimental period extending from the 1st to the 42nd day.

The results of this experiment, presented in Table III, show that the factor in yeast was more stable to autoclaving than that in hexane-extracted alfalfa. Extraction with 50 per cent methanol removed most, but not all, of the factor from yeast. Hot methanol did not extract any of the factor from hexane-extracted alfalfa, while warm water removed the greater part of it.

*Solubility in Solvents of Varying Concentrations*—The solubility of the growth factor in ether and in varying concentrations of methanol, isopropanol, and acetone was next studied. Portions of 500 gm. of dried brewers' yeast were extracted five times with 2000 ml. portions of each solvent, the yeast being allowed to remain in the solvent overnight during each extraction. The extracts were concentrated under reduced pressure and were assayed with Diet 38. This diet was identical with Diet 1A except that the rice bran filtrate was replaced by the filtrate factor concentrate made from whey. In this experiment, the assays were made with chicks in groups of seven to eight, and covered a period of 2 weeks preceded by a 2 week depletion period on Diet 38.

Table IV presents the growth results obtained by the use of various solvents. From these data it can be seen that ether, acetone, isopropanol, and 75 per cent isopropanol did not extract appreciable amounts of the growth factor, these extracts showing no potency when fed at levels equivalent to 7.5 per cent of yeast, which is at least 2.5 times the amount of yeast required for maximum growth. Methanol and 75 per cent acetone extracted some of the factor, and 75 per cent methanol extracted larger amounts.

TABLE III

*Solubility of Growth Factor in Water and Methanol and Its Stability to Autoclaving; Assays Made with Diet 1A*

Supplement	Weight, 42 days	Mortality, 42 days
	gm.	per cent
None.....	171	82
7.5% dried yeast.....	428	0
7.5% autoclaved yeast.....	469	0
50% methanol extract of yeast, equivalent to 7.5% yeast.....	449	0
7.5% yeast residue from 50% methanol extraction....	314	9
5% hexane-extracted alfalfa.....	417	0
10% " " ".....	443	0
50% autoclaved hexane-extracted alfalfa.....	176	45
Water extract of " " equivalent to 10% alfalfa.....	365	18
10% hexane-extracted alfalfa residue from water extraction.....	220	64
Methanol extract of hexane-extracted alfalfa, equivalent to 10% alfalfa.....	215	37
50% methanol extracted, hexane-extracted alfalfa, equivalent to 7.5% alfalfa.....	481	0

*Behavior toward Adsorbents, and Stability to Acid and Alkali—*

A further study of the properties of the factor was made with a 50 per cent methanol extract of yeast as the source of the factor, the diet and method of assay described in the previous experiment being used.

The 50 per cent methanol extract of yeast was prepared by extracting 3 kilos of dried yeast three times with 12 liter portions of 50 per cent methanol. The combined extracts were concentrated under reduced pressure until 1 ml. of the concentrate was

equivalent to 5 gm. of the original yeast. A 135 ml. portion of this extract was diluted to 1500 ml. and brought to pH 1.0 with hydrochloric acid. 150 gm. of English fullers' earth were added, the mixture shaken, the earth filtered off, and the filtrate treated a second time with 75 gm. of fullers' earth. The combined adsorbates were washed twice with dilute hydrochloric acid solution at pH 1.0. The filtrate and washings were neutralized with sodium hydroxide and concentrated under reduced pressure. A similar adsorption was made at pH 7.0 with the same relative amounts of fullers' earth. Part of the fullers' earth adsorbate, prepared at pH 1.0, was dried and fed directly. Another part,

TABLE IV

*Solubility of Growth Factor in Solvents of Varying Concentrations;  
Assays Made with Diet 38*

All extracts fed at a level equivalent to 7.5 per cent of dried yeast.

Supplement	Gain, 14 to 28 days
	gm.
None.....	44.9
3% dried yeast.....	140.2
Ether extract of yeast.....	54.6
Acetone " " " .....	50.3
75%* acetone extract of yeast.....	76.3
Isopropanol " " " .....	50.8
75%* isopropanol extract of yeast.....	59.3
Methanol extract of yeast.....	94.2
75%* methanol extract of yeast.....	116.2

\* By volume.

equivalent to 225 gm. of yeast, was eluted twice with 400 ml. portions of a 1:1:4 mixture of pyridine, methanol, and water. The combined eluates were concentrated under reduced pressure until the odor of pyridine could no longer be detected.

Adsorptions were also made with activated charcoal at pH 1.0 and 8.0. 45 ml. of the yeast extract, equivalent to 225 gm. of yeast, were diluted to 550 ml. with water, and concentrated hydrochloric acid added to bring the pH to 1.0. 20 gm. of nuchar were added, the mixture shaken, filtered, and the filtrate treated a second time with 10 gm. of nuchar. The combined adsorbates were washed twice with acidulated water at pH 1.0. The filtrate

and washings were neutralized with sodium hydroxide solution and concentrated under reduced pressure. A similar adsorption was made at pH 8.0.

The stability of the factor to acid and alkali was also determined. 42 ml. of the yeast extract were diluted to 270 ml. with H<sub>2</sub>O, sufficient sodium hydroxide was added to bring the pH to 11.0, and the mixture refluxed for 30 minutes. The solution was neutralized and concentrated under reduced pressure. Another portion of the extract was brought to pH 1.7 with hydrochloric acid, refluxed for 30 minutes, neutralized, and concentrated.

TABLE V

*Behavior of Growth Factor toward Adsorbents and Its Stability to Acid and Alkali; Assays Made with Diet 38*

Supplement	Gain, 15 to 29 days
	<i>gm.</i>
None.....	51.6
3% dried brewers' yeast.....	123.7
50% methanol extract of yeast*.....	97.6
50% " " " " †.....	99.2
Fullers' earth filtrate (pH 1.0)†.....	36.2
" " " (" 7.0)†.....	85.2
" " adsorbate†.....	98.0
Pyridine eluate of fullers' earth adsorbate†....	143.4
Charcoal filtrate (pH 1.0)†.....	63.0
" " (" 8.0)†.....	65.6
Yeast extract refluxed at pH 1.7†.....	111.8
" " " " 11.0†.....	121.9

\* Equivalent to 3.0 per cent yeast in the diet.

† Equivalent to 7.5 per cent yeast in the diet.

The growth results of this experiment, presented in Table V, show that the growth factor was readily adsorbed on fullers' earth at pH 1.0 and to a lesser extent at pH 7.0. The fullers' earth adsorbate prepared at pH 1.0 was potent when fed at a level equivalent to 7.5 per cent of dried yeast. Elution with pyridine solution removed the factor from fullers' earth. Activated charcoal removed the factor from the yeast extract at pH 1.0 and also at pH 8.0. Refluxing for 30 minutes at pH 1.7 and 11.0 did not destroy the factor.

## DISCUSSION

The growth factor concerned in these experiments cannot be identified with any of the following. Riboflavin and the filtrate factor were adequately added to the basal diet in concentrates of known potency. The thiamine (0.2 mg. per 100 gm.) contained in the basal ration was ample, as Arnold and Elvehjem (2) have found that 0.1 mg. per 100 gm. of diet are adequate for growth. The soy bean oil furnished adequate amounts of vitamin K and some gizzard factor. The high potency sardine oil furnished an excess of vitamins A and D. Nicotinic acid had no effect on growth when fed at a level of 0.20 mg. per 100 gm. of diet. The other factors which have been described as being required by the chick are the antiencephalomalacia factor (3) and vitamin B<sub>4</sub> (4, 5). The antiencephalomalacia factor, being fat-soluble, cannot be identical with the unknown growth factor in question. That vitamin B<sub>4</sub> cannot be identified with the unknown growth factor is indicated by the following considerations. According to the assays of Kline, Bird, Elvehjem, and Hart (4), the minimum protective level of yellow corn was 40 per cent. However, in Diet 2, 55 per cent of yellow corn did not furnish enough of the unknown growth factor. Furthermore, a deficiency of vitamin B<sub>4</sub> in chicks, according to these authors, produces paralysis. However, few cases of paralysis have been observed in chicks on polished rice diets and the sporadic cases that do occur appear as frequently on diets containing amounts of the growth factor adequate for maximum growth as on the basal ration. This strongly suggests that the growth factor with which we are dealing is not the antiparalytic one.

The question arises whether this growth factor is vitamin B<sub>6</sub>. The following facts suggest that the two factors are distinct. Dann (6) found yellow corn to be a fairly rich source of vitamin B<sub>6</sub>, 1 gm. of yellow maize containing at least 1 day's dose of this vitamin. However, 55 per cent of yellow corn did not furnish enough of the growth factor for chicks. György (7) found cane molasses to be a rich source of vitamin B<sub>6</sub>, but 5 per cent of dried Hawaiian cane molasses had no effect on the growth of the chicks.

As the factor here concerned seems to be distinct from other nutritional factors thus far described, it is suggested that, until



a chemical name based on structure can be assigned, it be tentatively called Factor U.

#### SUMMARY

Evidence has been presented showing that chicks require a dietary factor for growth not identical with vitamins A, D, K, thiamine, riboflavin, filtrate factor, vitamin B<sub>4</sub>, and the anti-encephalomalacia factor.

The growth factor is present in large amounts in alfalfa, middlings, wheat bran, and yeast, to a lesser extent in corn, and in very small amounts in polished rice.

The factor is insoluble in ether, acetone, and isopropanol, but soluble in water and in mixtures of water and methanol. This growth factor can be adsorbed on fullers' earth and on activated charcoal, and can be eluted from the fullers' earth adsorbate by a 1:1:4 mixture of pyridine, methanol, and water.

Autoclaving yeast did not destroy the growth factor, while autoclaving alfalfa did destroy it. Refluxing in acid and in alkali media did not destroy the growth factor present in an extract of yeast.

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# CHEMICAL STUDIES ON THE GONADOTROPIC HORMONE OF PREGNANT MARE SERUM

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The purposes of a chemical study of the gonadotropic hormone of pregnant mare serum were essentially the same as those which prompted us to study three other protein hormone preparations; *viz.*, insulin (1), prolan (2), the pituitary gonadotropic fractions (3), and the prolan contaminant, callicrein (4).

## EXPERIMENTAL

*Preparation*—The starting material was the sterile pregnant mare serum of the Cutter Laboratories. Two types of preparations were used. One, which was relatively crude, represented the 50 per cent alcohol-soluble, 80 per cent alcohol-insoluble fraction obtained at pH 7.4. By reprecipitating this preparation at pH 6.0 and discarding the 50 per cent alcohol-insoluble material, further purification (20-fold) was attained. The final alcohol precipitates were washed with alcohol and ether and then air-dried. 0.1 mg. produced 200 to 300 per cent ovarian hypertrophy.

Evans *et al.*, (5) describe a separation of the crude mare serum into two distinct fractions, and postulate the existence of a separate interstitial cell-stimulating hormone, which in the male is regarded as stimulating the production of the steroid responsible for seminal vesicle development. It was originally planned to study the reactions of these two fractions to chemical reagents, but since a highly purified mare serum preparation supplied us by the Cutter Laboratories failed to separate by the ammonium sulfate fractional precipitation procedure of Evans into the two fractions, and since material fractionated directly from mare serum is too crude for chemical studies, the project was abandoned.

*Ammonium Sulfate Fractionation*—A solution of 1000 units (Cole and Saunders (6)) of mare serum preparation containing 0.45 mg. of nitrogen in 5 cc. was mixed with an equal volume of saturated ammonium sulfate solution at room temperature. The pH was adjusted to 7.0 with 0.1 N NaOH with brom-thymol blue as indicator, and a volume of ammonium sulfate solution equal to the alkali used was added. The precipitate was removed by centrifugation and washed with half saturated ammonium sulfate solution. The filtrate was raised to three-quarters ammonium sulfate saturation and the pH was adjusted to 5.0 with methyl red as the indicator. A correction for the volume of acid added was made. The precipitate which formed was removed by centrifugation. Six pairs of female and five pairs of male rats were used for the assay at one dosage level. For the precipitate at half saturation, the mean organ weights were 28 mg. for ovaries, 117 mg. for uterus, 21 mg. for seminal vesicles, and 83 mg. for prostate. For the precipitate at three-quarters saturation, the mean organ weights were 24 mg. for ovaries, 94 mg. for uterus, 17 mg. for seminal vesicles, and 81 mg. for prostate. The control values for these organs are respectively 12, 16, 9, and 53 mg. In each case the increase in seminal vesicle weight parallels the increase in ovarian and uterine weight and there is no evidence for the separation of an interstitial cell-stimulating from a follicle-stimulating hormone.

*Assay*—In order to minimize the influence of the normal variation in response, each chemically treated preparation was assayed simultaneously with an aliquot of untreated preparation, which was at the same pH as the treated material. Litter mate, immature, 22 to 23 day-old rats were used as the test animals. Since the response to pregnant mare serum is not greatly influenced by divided dosage, a single injection was made subcutaneously and the rats sacrificed 4 days later. In chemical studies, the single injection is advantageous because it can be made at the end of the reaction period, without readjustment of pH or removal of excess reagent.

As a standard, ovarian, uterine, and seminal vesicle weights were ascertained for series of rats injected with the untreated material at four different dosage levels. Since the results for ovaries and uteri confirm those of Cole and Saunders (6) and Cart-

land and Nelson (7), they have not been included in the present report. In a preliminary assay for testing and comparing the activity of treated and untreated material, four pairs of litter mates were used. When 90 per cent or more inactivation had

TABLE I  
*Effect of Various Reagents upon Pregnant Mare Serum Hormone*

Mare serum powder	Reaction mixture	Reagent and reaction environment	Per cent recovery by rat assay
mg.			
14	7	35 mg. $\text{CH}_3\text{COOH}$ , pH 4.0, 24 hrs.	100
1.0	2.7	24 " " 24 hrs.	75
14	7	1.4 cc. saturated $\text{Na}_2\text{BO}_3$ , pH 10.0, 24 hrs.	100
12	6	30 mg. $\text{CH}_3\text{COOH}$ , 0.4 cc. 0.1 N I, pH 4.0, 3 hrs.	100
12	6	0.25 cc. 0.1 N I, pH 7.4, 24 hrs.	25
12	6	120 mg. $\text{NaHCO}_3$ , 0.25 cc. 0.1 N I, 1 hr.	0
12.5	3	50 " $\text{CH}_3\text{COOH}$ , 30 mg. $\text{H}_2\text{O}_2$ , 24 hrs.	50
1.0	4	10 " $\text{NaHCO}_3$ , 30 mg. $\text{H}_2\text{O}_2$ , 1 hr.	20
1.0	4	11 " $\text{CH}_3\text{COOH}$ , 5 mg. $\beta$ -naphthoquinone sulfonate, 1 hr.	25
0.5	1.5	10 mg. $\text{NaHCO}_3$ , 5 mg. $\beta$ -naphthoquinone sulfonate, 1 hr.	0
12		42 mg. $\text{NaHCO}_3$ , 42 mg. $\beta$ -naphthoquinone sulfonate, 24 hrs.	0
12.5	3	50 mg. $\text{CH}_3\text{COOH}$ , 30 mg. $\text{HCHO}$ , 24 hrs.	50
0.75	2.2	20 " $\text{NaHCO}_3$ , 40 mg. $\text{HCHO}$ , 3 hrs.	50
0.75	2.2	20 " $\text{CH}_3\text{COOH}$ , 40 mg. $\text{HCHO}$ , 3 hrs.	50
0.5	2	10 " " 5 " $\text{NaNO}_2$ , 1 hr., 0°	100
12.5	3	50 " " 21 " " 24 hrs., 20°	0
0.6	6	120 mg. $\text{NaHCO}_3$ , 26 mg. $(\text{CH}_3)_2\text{SO}_4$ , 0.5 hr.	75
0.6	6	120 " " 26 " " 48 hrs.	25
14	7	40 mg. $(\text{CH}_3\text{CO})_2\text{O}$ , 24 hrs., 0°	25
14	7	40 " " 200 mg. $\text{NaHCO}_3$ , 24 hrs., 0°	0
1.0	3	40 " $\text{CH}_3\text{COOH}$ , 11 mg. diazobenzene sulfonate, 1 hr., 0°	20
1.0	3	40 mg. $\text{NaHCO}_3$ , 11 mg. diazobenzene sulfonate, 1 hr. 0°	0

occurred, this number sufficed, as the uterine response is very sensitive to minimal dosage. The results in Table I have been reported to the nearest 25 per cent, since in an assay in which six pairs of rats are used, greater accuracy cannot be claimed.

*Chemical Reactions*—The amounts of pregnant mare serum powder and the various chemical reagents, the reaction volume, and other reaction conditions are given in Table I. In the studies on insulin and pituitary gonadotropic preparation, excess of reagent was separated from reaction products in most cases by processes known to separate the original material. It was necessary to assume that the properties of the reaction products were not sufficiently changed to interfere with their recovery. In the present studies with pregnant mare serum hormone and in the previous experiments with prolان, no separation of the reaction products from excess reagent was made. The amounts of pregnant mare serum hormone and prolان required for physiological assay are so minute, because of the high physiological activity, that excess of reagent did not interfere with the assay.

### *Results*

At a mildly alkaline pH, iodine,  $\beta$ -naphthoquinone sulfonate, acetic anhydride, and diazobenzene sulfonate all caused complete inactivation of the mare serum preparation. Under the same conditions hydrogen peroxide and dimethyl sulfate caused only partial inactivation. At a mildly acid pH,  $\beta$ -naphthoquinone sulfonic acid, acetic anhydride, and diazobenzene sulfonic acid caused partial inactivation, in contrast to the total inactivation produced at a slightly alkaline pH. The action of iodine was governed by the pH, none occurring in acid solution. In dilute solution and at 0°, nitrous acid produced no inactivation. At room temperature and on prolonged action complete inactivation occurred.

Cartland and Nelson (8), using a procedure similar to that used by us (3) for the anterior lobe fraction, have reported upon the action of formaldehyde upon the pregnant mare serum product. In a 3 per cent solution, they obtained 84 per cent inactivation in 3 hours. No qualitative changes in the ovarian response were noted. With a lower formaldehyde concentration, we obtained less inactivation.

In the present study as in the studies on prolان, no structural groups or nuclei can be characterized as responsible for the physiological activity. Certain characteristic trends, nevertheless,

are revealed: (1) the relative stability to dimethyl sulfate, formaldehyde, and nitrous acid in contrast to the extreme sensitivity to diazonium compounds, to acylation, and to reaction with naphthoquinone sulfonate; (2) a more pronounced tendency to be inactivated by reagents in mildly alkaline solution (acylation,  $\text{H}_2\text{O}_2$ , I, naphthoquinone sulfonate, diazobenzene sulfonate) than in mildly acid solution.

#### DISCUSSION

The results of the foregoing experiments indicate a marked similarity in the stability and reactivity of the pregnant mare serum hormone, prolan, and the pituitary sex hormone to a wide range of chemical reaction. No significant difference could be detected between prolan (urine of pregnancy) and the pregnant mare serum hormone. The pituitary preparation was more sensitive to methylation. The physiologic response to prolan and the pregnant mare serum product is not greatly affected by divided dosage, while the response to the pituitary preparation is affected some 2000 per cent by divided dosage or delayed resorption (9). This constitutes the greatest difference yet observed for these substances, and if there is any correlation between this observation and the relative stability to methylation, the reason is as yet not apparent.

Our complete chemical studies for the gonadotropic substances obtained from the three sources and covering a wide range of reactions have in no instance indicated a separation by a selective inactivation of one gonadotropic fraction from another. There is probably a series of gonadotropic substances as there are estrogens differing but slightly in chemical structure, *which all stimulate the same cells*, but in varying degree. No clear evidence has been obtained by us that there are separate interstitial cell-stimulating, follicle-stimulating, and luteinizing hormones. When minimal threshold doses are given, all the gonadotropic hormones produce the same result, liberation of estrogenic substance as measured by uterine hypertrophy, without detectable cytological differences in the ovary.

We are indebted to Dr. D. H. Wonder of the Cutter Laboratories for a generous supply of pregnant mare serum.

## SUMMARY

1. Pregnant mare serum hormone in mildly alkaline solution is more than 90 per cent inactivated by acetylation, reaction with  $\beta$ -naphthoquinone sulfonate, coupling with diazobenzene sulfonate, and by iodination. In mildly acid solution these reagents produce less or no inactivation.

2. Pregnant mare serum hormone is relatively stable to moderate excess of nitrous acid, dimethyl sulfate (even in alkaline solution), and to formaldehyde.

3. In the sixteen different conditions of reaction environment studied, pregnant mare serum hormone responded in a manner analogous to the response of prolactin as previously reported.

4. A highly purified hormone preparation of pregnant mare serum failed to separate into fractions (interstitial cell-stimulating and follicle-stimulating hormones) when subjected to ammonium sulfate precipitation.

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## CONVERSION OF URONIC ACIDS INTO CORRESPONDING HEXOSES

### VII. CATALYTIC REDUCTION OF METHYL ESTER OF HEXA- METHYL METHYLGLYCOSIDE OF ALDOBIONIC ACID (OF GUM ARABIC) TO METHYLGLYCOSIDE OF HEXAMETHYL 6-GLUCOSI- DOGALACTOSE. FURTHER METHYLATION TO METHYLGLYCO- SIDE OF HEPTAMETHYL 6-GLUCOSIDOGALACTOSE

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The objects of the investigation to be presented in this communication have been outlined in previous communications.<sup>1</sup> The matter of methylation of aldobionic acids is still beset with difficulties, illustrated best by the experience of Haworth *et al.*<sup>2</sup> in their work on the structure of the aldobionic acid of gum arabic. The conversion of the methylated aldobionic acid to a methylated biose is a new undertaking.

The conditions of methylation in use in our laboratory permit the methylation of aldobionic acids as readily as the methylation of bioses and with similar yields. Advantage was taken of the best features of the procedures of Haworth,<sup>3</sup> and of West and Holden.<sup>3</sup> The success of the methylation depends on very vigorous stirring. When 15 gm. lots are used, the reaction is completed in 5 hours, the yield being from 80 to 90 per cent of the weight of the starting material. An additional quantity can be obtained from the aqueous residue of the first methylation product. When the aldobionic acid is as readily prepared as is the one from

<sup>1</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **125**, 345 (1938). Levene, P. A., Tipson, R. S., and Kreider, L. C., *J. Biol. Chem.*, **122**, 199 (1937).

<sup>2</sup> Challinor, S. W., Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 258 (1931).

<sup>3</sup> West, E. S., and Holden, R. F., *J. Am. Chem. Soc.*, **56**, 930 (1934).



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gum arabic, the remethylation of the aqueous residue yields scarcely enough material to justify the operation.

In the methylation of bioses and often even of monoses, the operation with dimethyl sulfate is generally followed by remethylation by the Purdie method. In the case of aldobionic acids it was found advantageous to methylate the carboxyl group with diazomethane preliminary to remethylation by the Purdie method. The product obtained on one remethylation is then readily distillable as the methylglycoside of the hexamethyl aldobionic methyl ester.

The reduction of the  $\text{COOCH}_3$  group to a primary alcoholic group is accomplished by treating the methanol solution of the substance over copper chromite catalyst for 3 hours at  $175^\circ$  in a hydrogen atmosphere at a pressure of 3500 pounds per sq. inch. The solution, freed from the catalyst, on concentration yields a crystalline product, the methylglycoside of hexamethyl 6-glucosidogalactose, and a small residue of syrupy material which has not been thoroughly analyzed. In one experiment, when the heating was prolonged overnight and the yield of the syrupy material was considerable, it could be separated into two fractions, one boiling at  $120\text{--}180^\circ$ ,  $p = 0.2$  mm., the other at about  $220\text{--}240^\circ$ ,  $p = 0.2$  mm. The latter had the composition of a methylglycoside of hexamethyl 6-glucosidogalactose.

The crystalline hexamethyl methylbioside is readily recrystallized from ether. One remethylation by the Purdie method was not sufficient to accomplish complete methylation of the bioside but the two components could be readily separated by virtue of the differences of their solubilities in ether. The fraction insoluble in cold ether consisted mostly of the hexamethyl methylbioside and the soluble fraction of heptamethyl methylbioside. For purification the latter was recrystallized from pentane and found to have properties identical with those previously recorded by Levene and Tipson<sup>4</sup> for the heptamethyl methylbioside obtained on exhaustive methylation of synthetic 6- $\beta$ -glucosidogalactose.

Thus there can be no doubt that the biose union in the aldobionic acid is identical with that in the synthetic disaccharide (namely,  $\beta$  configuration).

<sup>4</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **125**, 355 (1938).

## EXPERIMENTAL

*Methylation of Aldobionic Acid from Gum Arabic*—15 gm. of crystalline aldobionic acid dihydrate<sup>5</sup> were dissolved in 20 cc. of water and acetone was added to opalescence. A total of 133 cc. of dimethyl sulfate and 340 cc. of 30 per cent sodium hydroxide was used for the methylation which was completed in three stages.

1. 15 cc. of dimethyl sulfate were introduced into the flask with the bath maintained at 35–40°. Dimethyl sulfate was then allowed to drop into the flask at the rate of a drop in 3 seconds and the alkali at the rate of a drop in 2 seconds. At this rate the operation was continued for 30 minutes.

2. The temperature of the bath was raised to 50°. The rate of flow of methyl sulfate and of alkali was increased so that one-half of the total methyl sulfate and of the total alkali was added in 2½ hours. Warming at this temperature was continued, without further addition of reagents, for 15 minutes.

3. The temperature of the bath was raised to 70–75° and the remaining half of dimethyl sulfate and of the alkali was added so as to complete the operation in 90 minutes. Then the stirring was continued, the bath being maintained at the same temperature for an additional 30 minutes. The total time consumed for methylation was 5 hours. Extraction of the product was performed as usual. Yields varied from 11 to 13 gm.

A second methylation of the aqueous residue yielded from 1.5 to 3 gm. This operation, however, was so time-consuming that it was generally omitted, the aldobionic acid of gum arabic being so readily accessible.

Material obtained in this manner was not completely methylated. Before further methylation by the Purdie method it was allowed to stand not less than 5 hours with an excess of an ethereal solution of diazomethane. After remethylation by the Purdie method and prior to distillation, the yield was 12 gm. It was found advantageous prior to reduction of the  $-\text{COOCH}_3$  to purify the substance by distillation, as otherwise it frequently poisoned the catalyst. On slow distillation without a capillary tube at a pressure of 0.2 mm. the substance distilled at a bath temperature of

<sup>5</sup> Heidelberger, M., and Kendall, F. E., *J. Biol. Chem.*, **84**, 639 (1929).

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220–240° as a viscous yellowish liquid. Yield 11.4 gm.  $n_D^{20} = 1.4656$ .  $n_D^{25} = 1.4641$ .

$$\frac{1 \times 100}{2 \times 3.76} = -22.3^\circ \text{ (in absolute ethanol)}$$

3.306 mg. substance: 33.44 cc. 0.01 N  $\text{Na}_2\text{S}_2\text{O}_3$

5.295 " " : 10.000 mg.  $\text{CO}_2$  and 3.690 mg.  $\text{H}_2\text{O}$

$\text{C}_{27}\text{H}_{36}\text{O}_{12}$ . Calculated. C 51.3, H 7.8,  $\text{OCH}_3$  53.0

Found. " 51.5, " 7.8, " 52.3

*Catalytic Reduction of Methylglycoside of Hexamethyl Aldobionic Acid Methyl Ester*—11 gm. of the substance were dissolved in 100 cc. of methanol. 10 gm. of copper chromite were added and the mixture allowed to remain in the hydrogenation apparatus during 3 hours at 175° at a pressure of 3500 pounds per sq. inch. The apparatus was allowed to cool overnight and the filtrate was then concentrated to a thick sirup which soon crystallized. The substance was recrystallized from ether. Four crops of crystals were obtained on gradual spontaneous concentration. Total yield, 8.5 gm. The substance had a melting point of 140–141° (sharp).

5.600 mg. substance: 10.605 mg.  $\text{CO}_2$  and 4.150 mg.  $\text{H}_2\text{O}$

3.132 " " : 29.77 cc. 0.01 N  $\text{Na}_2\text{S}_2\text{O}_3$

$\text{C}_{19}\text{H}_{24}\text{O}_{11}$ . Calculated. C 51.8, H 8.2,  $\text{OCH}_3$  49.3

Found. " 51.8, " 8.4, " 49.3

The specific rotation of the substance was as follows:

$$[\alpha]_D^{25} = \frac{-2.25^\circ \times 100}{2 \times 3.54} = -31.8^\circ \text{ (in absolute ethanol)}$$

*Methylation of Methylglycoside of Hexamethyl 6-Glucosidogalactose to Methylglycoside of Heptamethyl 6-Glucosidogalactose*—2.5 gm. of the hexamethyl 6-glucosidomethylgalactoside were remethylated by means of dimethyl sulfate and 60 per cent alkali according to West and Holden<sup>2</sup> (second phase).

The chloroform extract was concentrated to dryness, giving a residue which was slightly yellow and which crystallized from a little ether and pentane. The first crystals, on recrystallization from petroleum ether, melted at 138° and hence were original material. The substance from the mother liquor, on concen-

tration and on repeated recrystallization from pentane, yielded 1.2 gm. of the methylheptamethyl derivative. It had a melting point of 73° (sharp) and its composition was as follows:

4.880 mg. substance:	9.430 mg. CO <sub>2</sub> and 3.660 mg. H <sub>2</sub> O
3.001 " "	: 31.91 cc. 0.01 N Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>
C <sub>20</sub> H <sub>38</sub> O <sub>11</sub> .	Calculated. C 52.8, H 8.4, OCH <sub>3</sub> 54.6
	Found. " 52.7, " 8.4, " 55.0

The specific rotation of the substance was as follows:

$$[\alpha]_D^{25} = \frac{-1.18^\circ \times 100}{1 \times 4.1} = -28.8^\circ \text{ (in absolute ethanol)}$$

Levene and Tipson<sup>4</sup> report a melting point of 75° and  $[\alpha]_D^{25} = -25.5^\circ$  (in absolute ethanol) for synthetic heptamethyl 6-glucosidomethylgalactoside.



## PROTEINOGENIC ALKYL ALCAMINES. II

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In a previous communication<sup>1</sup> we described the direct synthesis of amino alcohols and N-dimethylamino alcohols from esters of amino acids, by reduction in the appropriate solvent in an atmosphere of hydrogen over the copper chromite catalyst. It was expected that likewise, in solution in the higher normal alcohols, N-disubstituted alcamines would be formed. The formation of N-monosubstituted derivatives was expected from reduction in solution in isopropyl alcohol.

It has now been found that only in the solution of ethanol does an N-disubstituted derivative form as readily as in methanol. In solution of higher homologues the predominating product was the N-monosubstituted derivatives. A similar result was obtained when isopropyl carbinol was used as solvent. It should be emphasized, however, that the search for conditions favoring the formation of N-disubstituted derivatives of the higher homologues is not fully completed.

To date, success has been obtained in the formation of the N-dipropyl derivative of leucinol. More drastic conditions (an initial temperature of 250° and initial pressure of 3000 pounds per sq. inch) were required for this synthesis.

In view of the physiological importance of the phenylated alcamines it was found desirable to test the behavior of *dl*-phenyl-alanine methyl ester under the treatment described above. In agreement with the experience of Adkins,<sup>2</sup> it was found that the phenyl group was not hydrogenated. On the other hand, the transformation into an N-disubstituted alkamine proceeded in the same way as in the case of aliphatic amino acids.

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<sup>2</sup> Adkins, H., *Reactions of hydrogen*, Madison (1937).

## EXPERIMENTAL

*Synthesis of N-Diethyl-dl-Leucinol by Reduction of dl-Leucine Methyl Ester in Ethyl Alcohol Solution*—10 gm. of copper chromite catalyst were added to a solution of 8.5 gm. of *dl*-leucine methyl ester in 150 cc. of absolute ethyl alcohol. This mixture was placed in a high pressure hydrogenation apparatus at a pressure of 2500 pounds per sq. inch, and a temperature of 175°, the hydrogen pressure rising to 3600 pounds per sq. inch. After the reaction had proceeded during 7 hours at the latter temperature, the whole apparatus was allowed to cool for 16 hours.

The mixture was centrifuged for 30 minutes and the supernatant liquid filtered through a layer of charcoal. The centrifuged solids were washed with 200 cc. of warm absolute ethyl alcohol and then the washings were filtered through the above layer of charcoal. Dry hydrogen chloride gas was passed into the combined filtrates until they became acid to Congo red and the acid solution was concentrated under diminished pressure to a thick sirup. The last traces of hydrogen chloride and moisture were removed by repeated concentrations with dry benzene.

This sirup did not crystallize until crystal nuclei were obtained through the picrate. The picrate was converted into the hydrochloride as in the case of *N*-dimethyl-*dl*-leucinol hydrochloride. The sirupy hydrochloride was converted into the free base in the following manner.

The sirupy substance was dissolved in 25 cc. of water (cooled to 0°) and a cold solution of 8 gm. of sodium hydroxide in 20 cc. of water was added. This cold solution was extracted three times with 50 cc. portions of ether. The combined ether extracts were dried with anhydrous potassium carbonate and the ether distilled off at atmospheric pressure through a fractionating column. The product was dried for 10 minutes with several pellets of sodium hydroxide and then distilled at 105–107° at 15 mm. pressure. Yield 8.6 gm.

The substance is soluble in ether, chloroform, acetone, and alcohol but is insoluble in water and pentane. It has a composition agreeing with that calculated for an *N*-diethylaminohexanol (free base).

4.318 mg. substance: 10.995 mg. CO<sub>2</sub> and 5.185 mg. H<sub>2</sub>O  
 4.400 " " : 0.307 cc. N<sub>2</sub> (763.5 mm. at 25°)  
 C<sub>10</sub>H<sub>22</sub>ON. Calculated. C 69.38, H 13.40, N 8.07  
 Found. " 69.43, " 13.42, " 8.03

*Preparation and Decomposition of the Picrate of N-Diethyl-dl-Leucinol*—3 gm. of distilled N-diethyl-dl-leucinol were dissolved in 10 cc. of ether and an ether solution of 2.5 gm. of dry picric acid was added. After standing in the refrigerator for several days the product had completely crystallized. A constant melting point of 79–80° was reached after two recrystallizations from ethanol-ether-pentane. The substance had a composition agreeing with that calculated for the picrate of an N-diethylamino-hexanol.

4.814 mg. substance: 8.420 mg. CO<sub>2</sub> and 2.790 mg. H<sub>2</sub>O  
 C<sub>16</sub>H<sub>26</sub>O<sub>8</sub>N<sub>4</sub>. Calculated, C 47.76, H 6.51; found, C 47.69, H 6.49

The pure substance was then dissolved in the minimum of warm water and 15 cc. of concentrated hydrochloric acid added. The precipitated picric acid was filtered off and the filtrate extracted three times with benzene in order to remove the last traces of picric acid. The aqueous solution was concentrated to a dry sirup under diminished pressure and the last traces of hydrochloric acid removed by repeated concentrations with dry benzene. The product would not crystallize and it was therefore not analyzed, since the pure picrate had been used.

*Synthesis of N-Dimethyl-dl-Phenylalaninol by Reduction of dl-Phenylalanine Methyl Ester in Methyl Alcohol*—10 gm. of copper chromite catalyst were added to a solution of 6 gm. of dl-phenylalanine methyl ester in 100 cc. of absolute methyl alcohol. This mixture was then reduced for 7 hours and the product isolated in the manner described in the previous experiment. The substance distilled at a temperature of 110° and 0.4 mm. pressure. Yield 5.3 gm.

It had a composition agreeing with that calculated for an N-dimethylaminophenylpropanol.

5.616 mg. substance: 15.115 mg. CO<sub>2</sub> and 4.889 mg. H<sub>2</sub>O  
 C<sub>11</sub>H<sub>17</sub>ON. Calculated, C 73.70, H 9.57; found, C 73.39, H 9.53



*Synthesis of N-Mono-n-Propyl-dl-Leucinol Hydrochloride by Reduction of dl-Leucine Methyl Ester in n-Propyl Alcohol*—15 gm. of copper chromite catalyst were added to a solution of 9 gm. of dl-leucine methyl ester in 150 cc. of n-propyl alcohol. This mixture was then reduced for 10 hours at a temperature of 175° and an initial pressure of 3000 pounds per sq. inch.

The catalyst was removed as in the first experiment and the alcohol solution made acid to Congo red with dry hydrogen chloride gas. The product set to a solid mass upon the removal of the alcohol and hydrogen chloride by concentration under diminished pressure. The product was recrystallized from acetone. Total yield 9.3 gm. After two more recrystallizations from acetone the product melted at 117–118° and had a composition agreeing with that calculated for an N-monopropylaminohexanol hydrochloride.

5.124 mg. substance: 10.380 mg. CO<sub>2</sub> and 5.220 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>23</sub>ONCl. Calculated, C 55.24, H 11.35; found, C 55.24, H 11.39

*Preparation of Crystalline N-Mono-n-Propyl-dl-Leucinol (Free Base) from the Crude Hydrochloride*—The crude crystalline product obtained by removal of alcohol from the n-propyl alcohol reduction experiment was dissolved in 25 cc. of water (cooled to 0°) and a cold solution of 10 gm. of sodium hydroxide in 25 cc. of water was added. The solution was extracted with three portions of ether and these combined ether extracts were dried with anhydrous potassium carbonate.

The ether was distilled off at atmospheric pressure and the product dried for a few minutes with solid sodium hydroxide. The substance distilled at 125° and 20 mm. pressure and it immediately set to a semisolid crystalline mass. The product had a composition indicating the presence of both the mono- and di-propyl compounds. (Found, C 69.36, H 13.63.) The crystalline portion was dried from the non-crystalline fraction by dissolving in pentane, cooling to 0°, and then filtering off the crystals at 0°. This crystalline product melted at 52–53° and had a composition agreeing with that calculated for the N-monopropylaminohexanol.

4.125 mg. substance: 10.293 mg. CO<sub>2</sub> and 4.905 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>21</sub>ON. Calculated, C 67.92, H 13.33; found, C 68.04, H 13.30

The sirupy mother liquors undoubtedly contained the dipropylaminohexanol, in the light of the results obtained in the next experiment.

*Synthesis of N-Di-n-Propyl-dl-Leucinol Hydrochloride by Reduction of dl-Leucine Methyl Ester in n-Propyl Alcohol*—10 gm. of copper chromite catalyst were added to a solution of 5 gm. of dl-leucine methyl ester in 130 cc. of n-propyl alcohol. This mixture was then reduced for 12 hours at 250° and an initial pressure of 3000 pounds per sq. inch. The hydrochloride of the product was prepared in the manner described in the previous experiments.

The crude sirupy product was dissolved in 25 cc. of acetone, 25 cc. of ether were added, and the solution was allowed to stand for 1 week in the refrigerator. The crystalline material was filtered off and washed with cold acetone. Yield 2.3 gm. After two recrystallizations from acetone, the substance melted at 130–131° and had a composition agreeing with that calculated for an N-dipropylaminohexanol hydrochloride.

5.004 mg. substance: 11.100 mg. CO<sub>2</sub> and 5.310 mg. H<sub>2</sub>O  
C<sub>12</sub>H<sub>23</sub>ONCl. Calculated, C 60.58, H 11.86; found, C 60.49, H 11.87

*Synthesis of N-Monoisopropyl-dl-Leucinol Hydrochloride by Reduction of dl-Leucine Methyl Ester in Isopropyl Alcohol*—10 gm. of copper chromite catalyst were added to a solution of 7 gm. of dl-leucine methyl ester in 150 cc. of isopropyl alcohol. This mixture was reduced in the usual manner for 16 hours at a temperature of 175° and an initial pressure of 3000 pounds per sq. inch.

The product was isolated in the manner described in previous experiments. The crude product was dissolved in 60 cc. of acetone and cooled to 0°. Yield 6.6 gm. After two recrystallizations the substance melted at 92–93° and had a composition agreeing with that calculated for an N-monopropylaminohexanol hydrochloride.

4.218 mg. substance: 8.595 mg. CO<sub>2</sub> and 4.200 mg. H<sub>2</sub>O  
C<sub>9</sub>H<sub>19</sub>ONCl. Calculated, C 55.24, H 11.35; found, C 55.63, H 11.15

*Synthesis of N-Mono-n-Butyl-dl-Leucinol Hydrochloride by Reduction of dl-Leucine Methyl Ester in n-Butyl Alcohol*—15 gm. of

copper chromite catalyst were added to a solution of 8 gm. of *dl*-leucine methyl ester in 150 cc. of *n*-butyl alcohol. The reaction mixture was reduced as usual for 8 hours at 175° and an initial pressure of 3000 pounds per sq. inch.

The product was isolated in the usual manner and crystallized from acetone. Yield 8.1 gm. After two recrystallizations the product melted at 148–149° and had a composition agreeing with that calculated for an *N*-monobutylaminohexanol hydrochloride.

4.125 mg. substance: 10.293 mg. CO<sub>2</sub> and 4.905 mg. H<sub>2</sub>O

C<sub>16</sub>H<sub>24</sub>ONCl. Calculated, C 57.24, H 11.55; found, C 56.72, H 11.58

*Synthesis of N-Monoisobutyl-dl-Leucinol Hydrochloride by Reduction of dl-Leucine Methyl Ester in Isobutyl Alcohol*—15 gm. of copper chromite catalyst were added to a solution of 8 gm. of *dl*-leucine methyl ester in 150 cc. of *isobutyl* alcohol. The mixture was reduced for 16 hours at 3000 pounds per sq. inch and 175°.

The product was isolated as the hydrochloride as in previous experiments. Yield 7.0 gm. The substance melted at 151–152° after two recrystallizations from acetone and had a composition agreeing with that calculated for a monobutylaminohexanol hydrochloride.

4.323 mg. substance: 9.005 mg. CO<sub>2</sub> and 4.42 mg. H<sub>2</sub>O

C<sub>16</sub>H<sub>24</sub>ONCl. Calculated, C 57.28, H 11.55; found, C 56.80, H 11.44

## SOME OBSERVATIONS ON THE CHICK ANTI- DERMATITIS FACTOR\*

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Preliminary fractionation studies on the chick antidermatitis factor have been reported by Koehn and Elvehjem (1-3), by Lepkovsky and Jukes (4), and by Jukes (5). When it became apparent (6) that nicotinic acid was not the factor in liver extract which protected chicks against the dermatitis associated with heated grain rations, work was begun on the fractionation and concentration of this unknown substance. The factor in question has been designated the filtrate factor (4), but we prefer the more specific term, chick antidermatitis factor (6).

In this paper will be described the preparation of purified concentrates which are effective in the prevention of dermatitis in the chick. Experiments will also be described which indicate some of the chemical features of the protective substance.

### EXPERIMENTAL

*Assay Technique*—Day-old white Leghorn chicks were fed a heated grain ration supplemented with flavin and thiamine as previously described (6). Four chicks were used in each group for the assay. The fractions to be assayed were dissolved in water, spread on the basal ration, and dried at 40° before a fan. Each time a series of assays was begun, a control group on the

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basal ration alone was started. Preventive studies over a 5 week period were used throughout. The activity of the various fractions was determined by noting the increase in body weight of the birds during the assay period and especially by observing the absence or presence of dermatitic symptoms around the beak and eyes and on the legs (see Table I).

*Source of Vitamins*—The earlier fractionations were made on a liver extract powder by precipitating a large amount of inactive material from an aqueous solution of this powder through the addition of a mixture of alcohol and ether (2). Later on, through the kindness of Dr. David Klein, we secured a liver extract preparation from the Wilson Laboratories which was a by-product in the manufacture of the fraction used in pernicious anemia. This was a black, viscous sirup which contained 60 per cent of solid matter and was active at a level of 2 per cent. It was soluble in 92 per cent alcohol and will be designated in this paper Preparation A. The older procedure could not be satisfactorily applied to Preparation A because it contained a larger amount of the more soluble, inactive constituents of liver than the liver extract powder and hence the difficulties of purification of the vitamin were increased. Since the liver extract powder was rather expensive and since large amounts of Preparation A were available, it seemed advisable to develop methods of concentration applicable to it. While no increase in potency over that previously reported (3) has been obtained, some important properties of the vitamin which will undoubtedly aid in its final isolation have been determined.

*Ether Fractionation*—A 600 gm. portion of the liver extract was used in each fractionation. This was dissolved in 2 liters of water, treated with 200 gm. of fullers' earth, and the adsorbate was filtered and washed well. The fullers' earth was used merely to remove proteinaceous material which interfered in the other extraction steps. The filtrate and washings were concentrated under reduced pressure to about 1 liter, and adjusted to pH 6.5 to 7 with NaOH. The neutral solution was next extracted continuously with ether for 72 hours. The extract was practically inactive when fed at a level equivalent to 4 per cent of Preparation A. The residual aqueous solution was then acidified to thymol blue with HCl and extracted continuously with ether for

110 hours. The extract was active when fed at a level equivalent to 4 per cent of Preparation A. All of the potency was not re-

TABLE I

*Activity of Liver Fractions in Prevention of Chick Dermatitis*

Four chicks were used in each group.

Supplement	Equi- valent level	Average gain in 5 wks.	No. show- ing symp- toms
	per cent	gm.	
None.....	0	27	4
Preparation A.....	2	151	0
Ether extract from neutral solution.....	4	35	4
“ “ “ acid “ .....	8	106	2
“ residue.....	4	117	0
“ “ “ .....	4	78	2
Norit filtrate.....	4	39	4
“ eluate.....	4	102	0
CHCl <sub>3</sub> extract.....	4	13	4
“ residue.....	8	79	1
“ “ “ .....	4	123	0
Acetic acid-soluble fraction.....	4	79	0
“ acid residue.....	4	36	4
Ethyl acetate-soluble fraction.....	6	115	1
“ acetate residue.....	10	78	3
Benzene extract.....	8	41	4
“ residue.....	8	77	0
EtOH-soluble Zn salts of distillate.....	12	77	0
EtOH-insoluble “ “ .....	12	44	2
EtOH-soluble Ba “ .....	8	136	0
EtOH-insoluble “ “ .....	8	108	0
Acetylation mixture.....	6	83	4
Hydrolyzed acetyl extract.....	6	101	1
Ether-soluble acetyl extract.....	6	100	0
Ether-insoluble “ “ .....	6	53	3
CHCl <sub>3</sub> -soluble acetyl extract.....	12	135	0
CHCl <sub>3</sub> -insoluble “ “ .....	12	21	4
Ether-insoluble cold acetyl extract.....	8	102	1
Ether-soluble “ “ “ .....	8	116	1
Distillate at 0.1-0.01 mm.....	12	33	4
“ “ 10 <sup>-5</sup> mm.....	12	130	0
Distillation residue.....	15	18	4

moved by the ether, for the residual solution possessed some slight activity.

*Norit Adsorption*—The active ether extract was dissolved in 2 liters of water and 200 gm. of Pfanstiehl's norit A were added. The mixture was stirred for 30 minutes, filtered, and washed. The filtrate was inactive when fed at a level equivalent to 4 per cent of Preparation A.

It is not an easy matter to adsorb the vitamin on active norit. In order to get appreciable removal from the solution about half as much norit as there are solids must be used. In order to get complete removal, several times as much adsorbent as there are solids should be employed. If enough norit is used, the vitamin appears to be adsorbed in alkaline as well as in acid solution.

*Elution*—The norit adsorbate was suspended in 1 liter of 90 per cent alcohol, stirred for 15 minutes, and filtered. The filter cake was suspended in the same quantity of eluent, heated just to boiling, and filtered. Three more elutions with hot alcohol were performed. The combined eluates were then concentrated under reduced pressure to remove alcohol. The resulting solution was active when fed at a level equivalent to 4 per cent of Preparation A.

Mixtures of pyridine and alcohol, or acetone proved no more effective as an eluent than did alcohol alone. These solvents were not extensively used because of their greater cost. Pyridine and alcohol removed slightly more total dry weight, and acetone slightly less, than did alcohol.

*Chloroform Extraction*—The concentrated aqueous solution of the eluate was extracted continuously with  $\text{CHCl}_3$  for 24 hours. Approximately 40 per cent of the solids was found in the extract. However, the extract when fed at a level equivalent to 4 per cent of Preparation A was inactive and showed only very slight activity when fed equivalent to 8 per cent. The residual solution was active at a level equivalent to between 4 and 6 per cent of Preparation A. This  $\text{CHCl}_3$  residue contained 2 to 4 gm. of dry matter per 100 gm. of starting material. Fourteen such preparations have been made, and these figures for dry matter represent the maximum variation. When the solution was concentrated under reduced pressure to dryness, a mass of white crystals (succinic acid, m.p.  $184^\circ$ ) and a pale yellow oil were obtained.

*Solubility in Various Solvents*—The active constituent in the  $\text{CHCl}_3$  residue was soluble in glacial acetic acid, anhydrous acetone,

dry ethyl acetate, dioxane, or absolute alcohol. It was insoluble in dry ether, benzene, or chloroform. Only a small portion of the total dry matter was insoluble in the first solvents mentioned, so these were not used for routine preparation of concentrates. Some of the active substance remained in the ethyl acetate-insoluble portion, possibly owing to adsorption. Had it not been for this fact, the loss of dry matter by ethyl acetate extraction was great enough to justify the use of this solvent.

*Preparation of Metallic Salts*—Silver salts of the  $\text{CHCl}_3$  residue were prepared (by warming with  $\text{Ag}_2\text{O}$ ), separated with ethyl alcohol, and decomposed with  $\text{H}_2\text{S}$ . The activity was lost by this procedure, possibly owing to adsorption on  $\text{Ag}_2\text{S}$  or to oxidation by the  $\text{Ag}_2\text{O}$ .

Zinc salts were prepared (by warming the  $\text{CHCl}_3$  residue with excess  $\text{ZnCO}_3$ ), and separated with alcohol. The alcohol-soluble fraction contained most of the activity. Zinc salts of a highly active distillate (see below) were also prepared, and the activity was found to reside in the portion soluble in absolute alcohol.

The most advantageous salts for fractionation purposes were the barium salts. That portion which was soluble in absolute alcohol was found to contain the activity. When the barium was removed from this fraction with  $\text{H}_2\text{SO}_4$ , 275 mg. per 100 gm. of Preparation A remained. This material was active when fed at a level equivalent to 8 per cent of Preparation A. However, the routine use of a barium salt separation in the fractionation procedure is of doubtful value because the alcohol-insoluble portion was also active (at a level equivalent to 8 to 12 per cent of Preparation A). Three reprecipitations from absolute alcohol failed to remove the activity completely from this fraction.

*Acetylation*—The  $\text{CHCl}_3$  residue from 300 gm. of liver extract was dissolved in 50 cc. of acetic anhydride containing 2 cc. of pyridine, and the solution was heated for 1 hour on the steam bath. Excess reagents were removed by distillation under reduced pressure. The resulting product was entirely inactive at a level equivalent to 6 per cent of Preparation A.

A portion of the acetylation mixture was dissolved in alcohol, and the solution was saturated with gaseous ammonia. This solution was sealed in a tube and heated for 1 hour in a steam bath. Ammonia and alcohol were then removed under reduced pressure.



When this product was fed at a level equivalent to 6 per cent of Preparation A, it was found to be active.

Further experiments showed that the activity in the crude acetylation mixture was now soluble in benzene, dry ether, or chloroform. Before any fractions of the acetylated concentrate were fed, they were hydrolyzed in the manner described above.

It was also found that acetylation did not proceed to completion in the cold. The ether-insoluble portion of a concentrate treated with pyridine and acetic anhydride in the cold was found to be active when fed at a level equivalent to 8 per cent of Preparation A. This fraction contained 128 mg. of dry matter per 100 gm. of Preparation A. Here again, the same difficulty which made the barium salt fractionation of doubtful value also made the utility of this fractionation scheme questionable.

*Molecular Distillation*—Although the free vitamin appeared to be labile to dry heat, as shown by the method of preparation of the basal ration, it was found that the acetyl derivative could withstand 100° for prolonged periods. This finding led us to attempt fractional distillation in a high vacuum. The acetylated mixture was placed in a horizontal still similar to that used by Almquist (7), which was then evacuated to 0.1 to 0.01 mm. of mercury and maintained at 100°. Considerable viscous, colorless liquid distilled. When hydrolyzed, this distillate proved to be inactive. The residue was then transferred to the retort of a molecular still. The apparatus was evacuated to approximately  $10^{-5}$  mm. of mercury, and the retort was maintained at 100° for 8 hours. The material which had distilled proved to be active when fed at a level equivalent to 12 per cent of Preparation A, whereas the residue in the retort was inactive at a level equivalent to 15 per cent. Both fractions were hydrolyzed before they were fed.

The barium salt separation, acetylation, and distillation procedures were next combined. A colorless, ether-soluble liquid was obtained which was active (after hydrolysis) when fed at levels equivalent to 12 to 20 per cent of Preparation A. From 100 gm. of starting material, 108 mg. of distillate were obtained.

#### DISCUSSION

The results obtained from the various fractionation procedures enable some speculations to be made concerning the probable

chemical nature of the chick antidermatitis factor. The fact that it is extracted much more readily from acid solution by ether than it is from neutral solution suggests that it is acidic. Furthermore, the observation that the activity of concentrates was destroyed by acetylation, and that this activity was completely regained by hydrolysis, suggests that the vitamin must contain amino or hydroxyl groups. The former is eliminated by the fact (4) that nitrous acid does not affect the activity of concentrates.

The observation of several investigators (3, 4) that the vitamin is not adsorbed on charcoal can be most readily explained by the fact that they did not use sufficient adsorbent. With enough active charcoal the activity can be completely removed from aqueous solution.

#### SUMMARY

Considerable concentration and purification of the chick antidermatitis factor have been achieved by solvent fractionation, adsorption on norit, acetylation, and molecular distillation. The results of extraction with ether at various pH values have indicated that the factor is acidic in nature. Acetylation and hydrolysis experiments have demonstrated that the vitamin contains one or more hydroxyl or amino groups. It has been found that the free vitamin is soluble in ethyl acetate, dioxane, glacial acetic acid, and in the solvents previously reported to dissolve it, and that it is insoluble in ether, benzene, or chloroform. It is extracted from aqueous solution by ether, but not by the latter two solvents. When acetylated, however, it is also soluble in the three latter solvents. The acetyl derivative can be distilled at 100° and a pressure of approximately  $10^{-5}$  mm. of mercury. The barium and zinc salts are soluble in absolute alcohol.

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## CHANGES IN LIPID COMPOSITION OF NERVES FROM ARTERIOSCLEROTIC AND DIABETIC SUBJECTS\*

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The peripheral nerves from diabetic subjects were found to have a markedly lower lipid content than those from normal controls (1, 2). The degree of chemical change was correlated with the degree of vascular change, the level of nerve examined, and also with the clinical manifestations of the disease process. The degenerative lesions, studied by histopathological methods (3), were also most marked toward the periphery. The fact that the chemical and histological changes were most marked toward the periphery, in which the vascular change was greatest, suggested that the factor of arteriosclerosis was the most marked feature associated with the pathological changes in nerves from diabetic subjects.

Histopathological studies of peripheral nerves from extremities, amputated for arteriosclerotic gangrene, have shown a definite Wallerian degeneration and fibrosis (4). The changes were more pronounced in the distal portion of the nerve than in the proximal and were proportional to the degree of arteriosclerotic change in the vessels. It was suggested that ischemia was the fundamental factor in the neuropathology of arteriosclerosis and diabetes. The similarity of the neuropathological changes associated with diabetes and arteriosclerosis suggested a correlated study of the degree of chemical change in peripheral nerves from such subjects.

\* The experimental data in this paper are taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Department of Biochemistry, University of Rochester.

*Methods*

The control nerves were obtained from autopsies of subjects dying of various acute diseases. The possible effects of chronic wasting disease or old age on the chemical composition of the peripheral nerves were avoided. The series of nerves from diabetic subjects was obtained from amputations for gangrene. The arteriosclerotic series was obtained from amputations for arteriosclerotic gangrene.

The nerves were cleaned of extraneous tissue, and samples were weighed out for water and lipid determinations. The water content was obtained by difference after drying in an oven at 105°. The tissue sample for lipids was ground in sand and ex-

TABLE I  
*Lipid Composition of Nerves from Normal Subjects*

The values are given in per cent.

Nerve	No. of nerves		Phospholipid		Cholesterol		Cerebroside		Fat, moist weight	Water, moist weight
			Moist weight	Dry weight	Moist weight	Dry weight	Moist weight	Dry weight		
Femoral	23	Mean	4.58	13.36	1.45	4.37	1.59	5.36	9.05	65.8
		s.d.	0.59	2.23	0.31	0.79	0.49	1.98	4.68	5.0
Sciatic	3	Mean	4.34	13.58	1.47	4.59	1.45	4.52	9.03	68.0
		s.d.	0.28	3.56	0.11	0.94	0.34	1.65	3.15	5.2
Posterior tibial	12	Mean	3.87	13.13	1.30	4.37	1.25	4.24	8.04	68.3
		s.d.	0.53	4.00	0.24	1.51	0.48	2.21	3.38	6.9

tracted with boiling alcohol and ether. Aliquots of the alcohol-ether extract were taken for the lipid determinations. Phospholipid, determined by Bloor's method (5), represents the total oxidizable material in the acetone-insoluble fraction. Cholesterol, determined by Bloor's colorimetric method, represents the total sterol as measured by the Liebermann-Burchard reaction. Cerebroside, determined by Kimmelstiel's method (6), was calculated from the acid-hydrolyzable galactose in the alcohol-ether solution. Neutral fat represents the difference between the total fatty acids, determined by Bloor's oxidimetric procedure, and the phospholipid fatty acids, assumed to be 70 per cent of the phospholipid.

TABLE II  
Lipid Composition of nerves from *S. l.*

The values are given in per cent.

Nerve	No. of nerves	Phospholipid		Cholesterol		Cerebroside		Fat, moist weight	Water, moist weight
		Moist weight	Dry weight	Moist weight	Dry weight	Moist weight	Dry weight		
Sciatic	4	2.66	7.84	0.71	2.06	0.67	1.96	15.02	64.3
		0.81	3.23	0.15	0.64	0.20	0.77	6.37	4.6
		-38.7	-42.3	-51.7	-55.1	-53.8	-56.8	+66.5	-5.5
Posterior tibial	8	1.56	4.87	0.60	1.90	0.57	2.18	10.29	71.7
		0.56	1.65	0.16	0.62	0.16	0.90	5.60	6.8
		-61.5	-63.0	-53.8	-57.8	-54.4	-48.6	+28.0	+5.0

TABLE  
of Nerve Labels Sub.

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The values are given in per cent.

Nerve	No. of nerves	Phospholipid		Cholesterol		Cerebroside		Fat, moist weight	Water, moist weight
		Moist weight	Dry weight	Moist weight	Dry weight	Moist weight	Dry weight		
Results of present investigation									
Sciatic	4	Mean	1.82	4.69	0.55	1.43	0.91	2.34	61.0
		s.d.	0.45	1.21	0.14	0.43	0.65	1.68	3.1
		Change	-56.8	-65.5	-62.5	-68.9	-37.3	-48.2	-10.3
Posterior tibial	12	Mean	1.23	3.59	0.36	1.09	0.44	1.24	63.0
		s.d.	0.55	2.03	0.15	0.67	0.23	0.70	8.2
		Change	-68.2	-72.6	-72.4	-75.0	-64.8	-70.8	-7.7
Results of previous investigations (1, 2)									
Femoral	11	Mean	2.58	0.88	0.88	1.04	12.35		
		s.d.	1.03	0.31	0.31	0.48	8.74		
		Change	-43.6	-39.3	-39.3	-34.6	+36.5		
Sciatic	5	Mean	2.14	0.86	0.86	0.97	11.70		
		s.d.	0.77	0.22	0.22	0.16	2.98		
		Change	-50.7	-41.4	-41.4	-32.4	+29.8		
Posterior tibial	36	Mean	1.07	0.42	0.42	0.72	12.70		
		s.d.	0.39	0.19	0.19	0.32	7.62		
		Change	-72.4	-67.6	-67.6	-42.4	+58.0		

*Results*

Table I shows the means and standard deviations of the lipids, calculated on the moist and dry weight basis, in nerves from normal subjects. Although the posterior tibial nerves appear to have slightly lower average values for the various lipid fractions, these differences are not significant. The data show only a slight indication of a correlation of composition with the level of the nerve examined, the lower levels tending to have the lower quantity of lipid.

Table II shows the lipid composition of nerves from gangrenous limbs of arteriosclerotic subjects. Both the sciatic and posterior tibial nerves show extensive changes from the normal. The means of the phospholipid, cholesterol, and cerebroside are markedly lower in the abnormal nerves, while the neutral fat values are higher than normal. The water content did not change significantly. The percentage change from normal of the various lipid fractions is in about the same order of magnitude. The posterior tibial nerves show greater percentage change from the normal than the sciatic. This indicates a greater pathology of the distal nerves than the proximal.

Table III shows the means, standard deviations, and percentage change from normal of nerves taken from gangrenous limbs of diabetic subjects. The means of values reported in previous publications (1, 2) are also given. In both series of nerves there is a marked decrease of phospholipid, cholesterol, and cerebroside from the normal means and a considerable increase in neutral fat. The distal nerves, the posterior tibial, show greater percentage change from the normal than the proximal. The magnitude of change is similar to that of the arteriosclerotic nerves.

The various lipid fractions show percentage changes of similar magnitude. Each series of nerves demonstrates a greater degree of chemical change in the distal than in the proximal part of the peripheral nerves.

## DISCUSSION

The decrease of phospholipid, cholesterol, and cerebroside in the nerves of arteriosclerotic and diabetic subjects is probably the result of autolytic, enzymatic processes in the degenerating nerves. The fatty acids, arising from the decomposition of



phospholipid, appear in the increased neutral fat fraction. However, the wide variation of neutral fat in both the normal and abnormal series is due to the presence of variable quantities of depot fat in the connective tissues of the nerve trunk.

A similar decomposition of the components of degenerating nerves was obtained by May (7). He found that, after cutting the peripheral nerves of the rabbit, lipid phosphorus, protein phosphorus, and total phosphorus decreased extensively and the split-products appeared in the increased amount of water-soluble phosphorus.

The similarity of the extent and quality of the chemical change in the two disease processes indicates a common factor operating in them. The greater extent of chemical change in the more distal parts of the nerves indicates that an inadequate blood supply and the concomitant anoxemia are the immediate causes of the decomposition of the lipid constituents, the structural elements of the nerve fibers.

#### SUMMARY

1. The phospholipid, cholesterol, cerebroside, neutral fat, and water contents were determined in the femoral, sciatic, and posterior tibial nerves from normal, diabetic, and arteriosclerotic subjects.

2. The femoral, sciatic, and posterior tibial nerves from normal subjects did not show significant difference in composition.

3. The peripheral nerves from diabetic and arteriosclerotic subjects showed marked decreases in phospholipid, cholesterol, and cerebroside, an increase in neutral fat, but no significant change in water content.

4. The posterior tibial nerves showed more extensive change from normal than the sciatic and the latter more than the femoral.

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# STUDIES ON THE SPECIFICITY OF CHOLINE ESTERASE

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The present investigation was undertaken to determine the relation between the structure of various substrates and the action of serum choline esterase upon them. The comparison of activities with different esters should take into account two factors: the hydrolytic velocities compared should be linear functions of time, and these velocities should be independent of substrate concentration. The first factor requires observations of the amount of ester split after certain intervals to establish the slope of the activity-time curve, and the second necessitates the determination of the initial velocities of hydrolysis with more than one substrate concentration to insure that further addition of substrate will not increase the speed of the splitting. In the past these points have been neglected in certain instances.

Previous work upon the specificity of choline esterase was carried out chiefly by Stedman and coworkers (1-4), Kahane and Lévy (5), Roépke (6, 7), and Vahlquist (8). Stedman's group collected evidence indicating that choline esterase is an enzyme distinct from lipase or simple esterase, and they rejected the implication of Vahlquist that this distinction was doubtful. Kahane and Lévy studied the hydrolysis of a variety of choline esters in connection with the effect of eserine in sensitizing tissues to the pharmacological action of these esters, and Roepke and associates investigated the affinity of the enzyme for a few substrates.

In the present work a systematic study was made of the effect on the enzyme action of variations in the structure of the acid component and the hydrocarbon portion of the alcohol component of the substrate. In several cases the effect of changing the associated anion was also observed. In addition the stereochemical specificity of choline esterase was investigated for the first time.

A few related compounds were studied in which there was no regular variation in structure.

#### EXPERIMENTAL

##### *Method*

In order to follow the course of the undisturbed hydrolysis, the gasometric method, with the Warburg apparatus, was adopted (Ammon (9)). 1 ml. of serum solution, prepared by diluting serum to the appropriate volume with bicarbonate-Ringer's solution, was used in the side arm of the Warburg vessel, while 3 ml. of substrate solution were placed in the main chamber. The substrate was dissolved in bicarbonate-Ringer's solution. The non-enzymatic control experiments contained 1 ml. of bicarbonate-Ringer's solution in the side arm.

The same stock of horse serum preserved over a few drops of  $\text{CHCl}_3$ , and stored at  $-6^\circ$ , was used for all of the measurements. From time to time it was tested against acetylcholine chloride but its activity remained practically constant for the duration of the investigation.

A mixture of 5 per cent  $\text{CO}_2$  and 95 per cent  $\text{N}_2$  saturated with water vapor was passed through the vessels for several minutes in the usual manner before starting the experiment. The reaction was carried out at  $30^\circ$  and manometer readings were recorded at suitable intervals, depending upon the speed of the reaction. In every case the activity-time curve was constructed and only the initial linear portion was used as a basis for comparison. The final concentrations of ester employed were between 0.5 and 1.0 per cent, but in every case a duplicate experiment with a higher substrate concentration was performed to observe whether a greater enzymatic activity could be elicited. Only data of maximum initial velocities were considered.

##### *Substrates*

Compounds I, II, and VII to XIX were provided through the courtesy of Dr. R. T. Major of Merck and Company, Rahway, New Jersey. The preparation and physical constants of these compounds have been described by Major and coworkers (10-12). Compounds XX to XXVI were kindly furnished by

Dr. A. Blankart of the Hoffman-La Roche Laboratories in Basel. Descriptions of these substrates have been given by Blankart (13). Compounds III to VI and XXVII were prepared by the author.

*Butyrylcholine Chloride, Compound III*— $\beta$ -Chloroethyl butyrate was prepared from ethylene chlorohydrin and butyryl chloride. The fraction of the ester distilling over at 79–80°, 11 to 12 mm. of Hg, was sealed in a glass tube with an excess of anhydrous  $(\text{CH}_3)_3\text{N}$ . After several weeks at room temperature the crystals of butyrylcholine chloride that had formed were washed with benzene and dried *in vacuo*. Cl, found, 17.2; theory, 16.9 per cent. On complete hydrolysis 100 mg. liberated 4.70 ml. of 0.1 N acid; theory, 4.77 ml.

*Isobutyrylcholine Chloride, Compound IV*— $\beta$ -Chloroethyl isobutyrate prepared as above, distilling at 66–70°, 8 to 9 mm. of Hg, was sealed with  $(\text{CH}_3)_3\text{N}$ . Analysis of the crystals gave Cl, found, 16.8; theory, 16.9 per cent. Hydrolysis of 100 mg. liberated 4.66 ml. of 0.1 N acid; theory, 4.77 ml.

*Palmitylcholine Chloride, Compound V*— $\beta$ -Chloroethyl palmitate (m.p. 44°) was prepared and treated with  $(\text{CH}_3)_3\text{N}$  as above. The platinum salt of palmitylcholine chloride, prepared with chloroplatinic acid, gave Pt, found, 18.0; theory, 17.9 per cent. By alcoholic alkaline hydrolysis 6.7 mg. of palmitylcholine chloride liberated 0.700 ml. of N/40 acid; theory, 0.710 ml.

*Benzoylcholine Chloride, Compound VI*—Choline chloride was refluxed with benzoyl chloride. The analysis of the product gave Cl, found, 14.5; theory, 14.6 per cent. Hydrolysis of 100 mg. liberated 4.20 ml. of 0.1 N acid; theory, 4.11 ml.

*Ethyl Ester of Betaine Chloride, Compound XXVII*—This compound was prepared from ethyl chloroacetate and  $(\text{CH}_3)_3\text{N}$ . Cl, found, 19.2; theory, 19.5 per cent; N, found, 7.63; theory, 7.71 per cent. Hydrolysis of 100 mg. liberated 5.46 ml. of 0.1 N acid; theory, 5.51 ml. The chloroplatinate gave Pt, found, 27.2; theory, 27.8 per cent.

The results of the studies on both the purely enzymatic hydrolysis and the alkaline hydrolysis alone are given in Tables I to IV. Fig. 1 shows the results of an experiment typical of those from which the data in the tables were derived. In order to facilitate comparison, the enzyme data presented in Tables I to

IV were all expressed as the hydrolysis in 30 minutes effected by 1 ml. of 10 per cent serum solution. The serum concentrations actually used, as well as the duration of the experiments, varied, depending upon the rate of hydrolysis. Usually 2 or 5 per cent rather than 10 per cent serum solutions and total reaction times from 30 to 300 minutes were employed. The data in Tables I to IV are extrapolations.

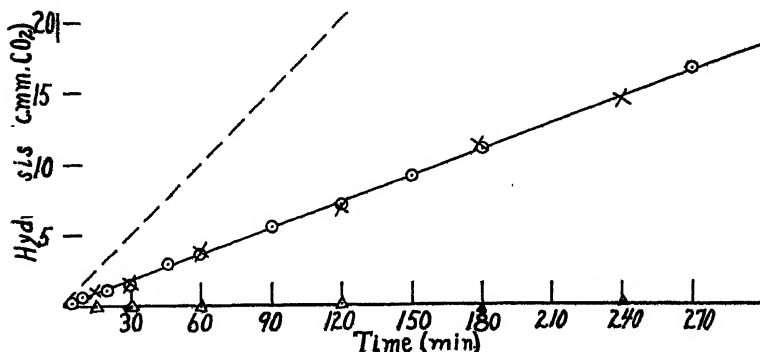


FIG. 1. Hydrolysis of optical isomers of acetyl- $\beta$ -methylcholine chloride. O, enzymatic hydrolysis of *d* form;  $\Delta$ , of *l* form;  $\times$ , of *dl* form. Corresponding hydroxyl ion hydrolysis of all isomers is indicated by the broken line.

#### DISCUSSION

It is apparent from Table I that increasing the normal chain length of the acid component of the ester from 2 to 4 carbon atoms resulted in a considerable increase in the enzymatic splitting, and a decrease in the alkaline hydrolysis, of these compounds. The relative hydrolyses observed were in fair agreement with the data of Easson and Stedman (3) for the corresponding bromides, and, in the case of the propionyl derivative, with the data of Kahane and Lévy (5) for the perchlorate. Further indication of the negative rôle played by certain anions associated with the ester was given by comparison of the data for Compounds XIX and XX (Table III). A comparison of the hydrolysis of Compounds IX and XII (Table II) and XXII and XXIII (Table III) lent additional evidence that increasing the length of the acid chain results in an increase of the enzymatic hydrolysis and a

decrease of the alkaline scission. However, this effect no longer held in the case of chains long enough to render the compounds water-insoluble, since the palmityl ester (Compound V, Table I) was barely affected by the enzyme.

When the acid component was kept constant, and changes were made in the structure of the choline portion of the molecule, the following observations resulted: A methyl group introduced into the  $\alpha$  position effected a drop in both enzymatic and non-

TABLE I  
*Hydrolysis of Esters of Choline*

Compound No.	Formula	Non-enzymatic hydrolysis extrapolated for 30 min. and 1 per cent substrate	Enzymatic hydrolysis extrapolated for 30 min. and 1 ml. 10 per cent serum
		c.mm. CO <sub>2</sub>	c.mm. CO <sub>2</sub>
I	(CH <sub>3</sub> ) <sub>3</sub> N(Cl)CH <sub>2</sub> ·CH <sub>2</sub> ·OCOCH <sub>3</sub> Acetylcholine chloride	13.0	225
II	(CH <sub>3</sub> ) <sub>3</sub> N(Cl)CH <sub>2</sub> ·CH <sub>2</sub> ·OCOC <sub>2</sub> H <sub>5</sub> Propionylcholine chloride	9.0	325
III	(CH <sub>3</sub> ) <sub>3</sub> N(Cl)CH <sub>2</sub> ·CH <sub>2</sub> ·OCOC <sub>3</sub> H <sub>7</sub> Butyrylcholine chloride	7.0	510
IV	(CH <sub>3</sub> ) <sub>3</sub> N(Cl)CH <sub>2</sub> ·CH <sub>2</sub> ·OCOCH(CH <sub>3</sub> ) <sub>2</sub> Isobutyrylcholine chloride	7.0	210
V	(CH <sub>3</sub> ) <sub>3</sub> N(Cl)CH <sub>2</sub> ·CH <sub>2</sub> ·OCOC <sub>15</sub> H <sub>31</sub> Palmitylcholine chloride	1.2*	1.2*
VI	(CH <sub>3</sub> ) <sub>3</sub> N(Cl)CH <sub>2</sub> ·CH <sub>2</sub> ·OCOC <sub>6</sub> H <sub>5</sub> Benzoylcholine chloride	4.0	5.4

\* A 0.2 per cent suspension was used.

enzymatic hydrolysis (Compound VIII, Table II). If the methyl was placed in the  $\beta$  position (Compound IX), a further drop in alkaline splitting was found but the decrease in enzymatic hydrolysis was extremely great. The hydrolysis of this compound was previously observed by Kahane and Lévy (5) and Roepke (7). Placing the group in the chain so that the ester linkage was further separated from the substituted amino radical (Compound XIX, Table III) effected increased non-enzymatic, but greatly decreased enzymatic hydrolysis as compared to the  $\alpha$ -methyl-substituted compound. Increasing the chain length of the

TABLE II  
Hydrolysis of Monoalkylcholine Esters

Compound No.	Formula	Non-enzymatic hydrolysis extrapolated for 30 min. and 1 per cent substrate	Enzymatic hydrolysis extrapolated for 30 min. and 1 ml. 10 per cent serum
		c.mm. CO <sub>2</sub>	c.mm. CO <sub>2</sub>
VII	$(\text{CH}_3)_3\text{N}(\text{C}_{17}\text{H}_{35}\text{COO})\text{CH}_2\cdot\overset{\text{CH}_3}{\text{CH}}\cdot\text{OCOCH}_3$ <i>dl</i> -Acetyl- $\beta$ -methylcholine stearate	42.0*	0.0*
VIII	$(\text{CH}_3)_3\text{N}(\text{I})\text{CH}_2\cdot\overset{\text{CH}_3}{\text{CH}}\cdot\text{OCOCH}_3$ <i>dl</i> -Acetyl- $\alpha$ -methylcholine iodide	7.0	156.0
IX	$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\cdot\overset{\text{CH}_3}{\text{CH}}\cdot\text{OCOCH}_3$ <i>dl</i> -Acetyl- $\beta$ -methylcholine chloride	5.0	3.6
X	<i>d</i> -Acetyl- $\beta$ -methylcholine chloride	4.8	3.6
XI	<i>l</i> -Acetyl- $\beta$ -methylcholine chloride	4.8	0.0
XII	$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\cdot\overset{\text{CH}_3}{\text{CH}}\cdot\text{OCOC}_6\text{H}_{11}$ <i>dl</i> -Caproyl- $\beta$ -methylcholine chloride	1.1	16.0
XIII	$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\cdot\overset{\text{CH}_3}{\text{CH}}\cdot\text{OCOC}_6\text{H}_4\text{NO}_2$ <i>dl</i> - <i>p</i> -Nitrobenzoyl- $\beta$ -methylcholine chloride	1.1	0.8
XIV	$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\cdot\overset{\text{C}_2\text{H}_5}{\text{CH}}\cdot\text{OCOCH}_3$ <i>dl</i> -Acetyl- $\beta$ -ethylcholine chloride	4.5	10.0
XV	$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\cdot\overset{\text{C}_3\text{H}_7}{\text{CH}}\cdot\text{OCOCH}_3$ <i>dl</i> -Acetyl- $\beta$ -propylcholine chloride	3.0	1.7
XVI	$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\cdot\overset{\text{C}_4\text{H}_9}{\text{CH}}\cdot\text{OCOCH}_3$ <i>dl</i> -Acetyl- $\beta$ -butylcholine chloride	2.0	3.2
XVII	$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\cdot\overset{\text{C}_5\text{H}_{11}}{\text{CH}}\cdot\text{OCOCH}_3$ <i>dl</i> -Acetyl- $\beta$ -amylcholine chloride	1.4	5.3
XVIII	$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\cdot\overset{\text{C}_6\text{H}_{13}}{\text{CH}}\cdot\text{OCOCH}_3$ <i>dl</i> -Acetyl- $\beta$ -hexylcholine chloride	1.3	7.4

TABLE III  
*Hydrolysis of Esters of  $\gamma$ -Homocholine and Its Derivatives*

Compound No.	Formula	Enzymatic hydrolysis extrapolated for 1 min. and 1 per cent substrate	
		c.m.m. CO <sub>2</sub>	c.m.m. CO <sub>2</sub>
XIX	(CH <sub>3</sub> ) <sub>3</sub> N(Cl)CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·OCOCH <sub>3</sub> Acetyl- $\gamma$ -homocholine chloride	8.0	12.0
XX	(CH <sub>3</sub> ) <sub>3</sub> N(I)CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·OCOCH <sub>3</sub> Acetyl- $\gamma$ -homocholine iodide		12.0
XXI	(CH <sub>3</sub> ) <sub>3</sub> N(I)CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·OCOCH(CH <sub>3</sub> ) <sub>2</sub> CH <sub>2</sub> CH=CH <sub>2</sub> Allylisopropylacetyl- $\gamma$ -homocholine iodide	3.0	0.0
XXII	(CH <sub>3</sub> ) <sub>3</sub> N(I)CH <sub>2</sub> ·C(CH <sub>3</sub> ) <sub>2</sub> ·CH <sub>2</sub> ·OCOCH <sub>3</sub> Acetyl- $\beta$ -dimethyl- $\gamma$ -homocholine iodide	2.3	11.0
XXIII	(CH <sub>3</sub> ) <sub>3</sub> N(I)CH <sub>2</sub> ·C(CH <sub>3</sub> ) <sub>2</sub> ·CH <sub>2</sub> ·OCOCH <sub>2</sub> ·CH(CH <sub>3</sub> ) <sub>2</sub> Isovaleryl- $\beta$ -dimethyl- $\gamma$ -homocholine iodide	1.6	38.0
XXIV	(CH <sub>3</sub> ) <sub>3</sub> N(I)CH <sub>2</sub> ·C(CH <sub>3</sub> ) <sub>2</sub> ·CH <sub>2</sub> ·OCOCH(OH)CH(CH <sub>3</sub> ) <sub>2</sub> $\alpha$ -Hydroxyisovaleryl- $\beta$ -dimethyl- $\gamma$ -homocholine iodide	4.0	4.0
XXV	C <sub>6</sub> H <sub>5</sub> <sub>2</sub> N(I)CH <sub>2</sub> ·C(CH <sub>3</sub> ) <sub>2</sub> ·CH <sub>2</sub> ·OCOCH(C <sub>2</sub> H <sub>5</sub> )CH <sub>2</sub> CB <sub>2</sub> =CH <sub>2</sub> Phenyl-2-bromoallylacetyl(3-hydroxy-2,2-dimethylpropyl) diethylmethyl ammonium	1.5*	2.6*



TABLE III—*Concluded*

Compound No.	Formula	Non-enzymatic hydrolysis extrapolated for 30 min. and 1 per cent substrate	Enzymatic hydrolysis extrapolated for 30 min. and 1 ml. 10 per cent serum
		<i>c.m.m.</i> CO <sub>2</sub>	<i>c.m.m.</i> CO <sub>2</sub>
XXVI	$  \begin{array}{c}  \text{CH}_3 \qquad \qquad \qquad \text{CH}_3 \qquad \qquad \text{C}_6\text{H}_5 \\  \diagdown \qquad \qquad \qquad \diagup \qquad \qquad \diagdown \\  \text{C}_2\text{H}_5 - \text{N}(\text{CH}_2\text{SO}_4)\text{CH}_2 \cdot \dot{\text{C}} \cdot \text{CH}_2 \cdot \text{OCOC} - \text{C}_6\text{H}_5 \\  \diagup \qquad \qquad \qquad \diagdown \qquad \qquad \diagup \\  \text{C}_2\text{H}_5 \qquad \qquad \qquad \text{CH}_3 \qquad \qquad \text{OH}  \end{array}  $ <p>Diphenylhydroxyacetyl(3-hydroxy-2,2-dimethylpropyl) diethylmethyl ammonium methyl sulfate</p>	4.2	0.0

$\beta$ -alkyl-substituted choline ester from 1 to 6 carbons, Compounds IX, XIV to XVIII, resulted in decreasing alkaline splittings. The enzymatic hydrolyses were in irregular order for the methyl, ethyl, and propyl compounds, but a regular increase in scission was found from the propyl- to the hexyl-substituted ester. It is to be noted that the introduction of two methyl groups into the chain of the  $\gamma$ -homocholine ester, Compound XXII, had hardly any effect upon the enzymatic hydrolysis. Furthermore, an  $\alpha$ -hydroxy group on the acid portion, Compound XXIV, effectively reduced the enzymatic splitting, possibly because of an affinity of the hydroxyl for the active group in the enzyme, similar to the case of liver esterase (14).

The stereochemical specificity of choline esterase was brought out by the data for the isomers of acetyl- $\beta$ -methylcholine, Compounds IX to XI. The inability of the enzyme to affect the levo member was interesting from the pharmacological view-point, since this isomer should then be destroyed more slowly *in vivo*. From this one would expect its pharmacological effect to be more prolonged than that of the racemic mixture now commonly used in clinical medicine. Prolongation of effect is often sought after in clinical applications. Apparently the levo compound has no affinity for the enzyme, since the splitting of the *dl* and *d* esters was identical.

Two related non-choline esters were included in the present investigation (Table IV). Ethyl acetate was studied simply to provide a comparison of the hydrolytic activities of the serum when the alkyl-substituted amino group was absent. Kahane and Lévy (5) reported the hydrolysis of the ethyl ester of betaine by serum. This result could not be duplicated by the author. The sample of the ester used in the present instance had a high grade of purity, as may be seen from the analyses given. Two separate preparations, made from separate lots of purified starting materials, both gave the result shown in Table IV.

There is no very satisfactory basis for an explanation of all of the results presented in the foregoing paragraphs. It might be

TABLE IV  
*Hydrolysis of Two Related Non-Choline Esters*

Compound No.	Formula	Non-enzymatic hydrolysis extrapolated for 30 min. and 1 per cent substrate	Enzymatic hydrolysis extrapolated for 30 min. and 1 ml. 10 per cent serum
		<i>c.mm. CO<sub>2</sub></i>	<i>c.mm. CO<sub>2</sub></i>
XXVII	(CH <sub>3</sub> ) <sub>3</sub> N(Cl)CH <sub>2</sub> COOC <sub>2</sub> H <sub>5</sub> Ethyl ester of betaine chloride	13.0	0.0
XXVIII	CH <sub>3</sub> COOC <sub>2</sub> H <sub>5</sub> Ethyl acetate	1.9	95.0*

\*No increase in activity was observed from 6.4 per cent final substrate concentration to saturated solution (8.6 per cent).

supposed that the decreased enzymatic hydrolysis observed when branching alkyl groups are introduced into either the acid (Compound IV) or choline components (Compounds VIII, IX, XXII) would be the result of steric hindrance around the ester linkage in a manner similar to the effect of steric hindrance around the hydroxyl groups of aliphatic alcohols with reference to their inhibiting effects upon liver esterase (14). However, this view is not borne out by the data in the  $\beta$ -alkyl-substituted choline esters (Compounds XIV to XVIII) or by the relatively slight reduction in the hydrolysis of Compound XXII as compared to XX. It is probable that steric hindrance with respect to the ester linkage is one, but certainly not the only, factor operating.

The surface effects, which undoubtedly play a part in the reaction between substrate and enzyme, are influenced by the size of the hydrocarbon portions of the ester molecules, and this factor may account for many of the effects observed. In general the surface activity increases as the hydrocarbon portion is enlarged and this should lead to greater facility of combination of enzyme and substrate. The enhanced enzymatic hydrolysis accompanying an increased length of the acid chain may be largely the result of this influence. Similar effects with other esterases have been observed, such as the increased hydrolysis by liver esterase of the  $\alpha$ -monoglycerides from monoacetin to monocaproin (15).

#### SUMMARY

Both enzymatic and non-enzymatic hydrolyses of a variety of choline esters have been measured in order to observe the effect of substrate structure upon the choline esterase activity.

Increased enzymatic scission accompanied lengthening of the acid chain, though with insoluble members such as palmitylcholine chloride and acetyl- $\beta$ -methylcholine stearate little or no effect was obtained.

Comparison of the present data with those reported by Easson and Stedman and Kahane and Lévy showed that interchanging the associated anion in the group, chloride, bromide, iodide, and perchlorate, had no demonstrable effect upon the hydrolysis.

Introduction of an  $\alpha$ -methyl group into the choline portion of the molecule effected a reduction in activity which was made more pronounced when the group was placed in the  $\beta$  position. Separation of the substituted amino radical from the ester link, as in the case of acetyl- $\gamma$ -homocholine, also decreased the enzymatic scission.

The effect of increasing the chain length of acetyl- $\beta$ -alkylcholine chloride from 1 to 6 carbon atoms was studied.

Little change was produced by adding two methyl groups to the center carbon atom in the  $\gamma$ -homocholine chain.

The enzymatic splitting was reduced by placing an  $\alpha$ -hydroxy group in the acid component.

For comparison ethyl acetate and the ethyl ester of betaine were included in the investigation. The latter was found to be unaffected by the enzyme, indicating that a requirement for the

choline esterase action is the presence of the substituted amino radical in only the alcohol component of the ester.

The optical isomers of acetyl- $\beta$ -methylcholine were tested and only the *d* member underwent enzymatic hydrolysis. Furthermore, the *l* ester showed no affinity for the enzyme.

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## STUDIES ON PHOTODYNAMIC ACTION

### II. THE FATE OF HEMATOPORPHYRIN AFTER PARENTERAL ADMINISTRATION

### III. THE INFLUENCE OF SENSITIZER ON PHOTOOXIDATION OF TISSUES

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#### *The Fate of Hematoporphyrin after Parenteral Administration*

In a previous communication (1) a study was made to determine the substances contained in body fluids which serve as acceptors and are oxidized during exposure to light in the presence of hematoporphyrin (Hp). It was found that the proteins and uric acid are photooxidized under these conditions and the influence of intensity of light, duration of exposure, concentration of substrate, concentration of sensitizer, pH, and temperature was studied. The present study deals with the fate of Hp injected by various routes into the body of living animals.

#### *Materials, Methods, and Technique*

Hp (Nencki) recrystallized twice was dissolved in saline, Ringer's solution, or phosphate buffer of pH 7 and injected intravenously, intraperitoneally, or subcutaneously.

*Apparatus*—Respirometers, water bath, temperature, and light control were identical with those described in the previous communication (1).

*Disappearance of Hp from Blood Stream after Intravenous Injection*—50 mg. of Hp per kilo of body weight were injected intravenously into a dog and samples of blood were taken after 5 minutes, 27 minutes, 45 minutes, 95 minutes, and 25 hours. Cumulative lymph was obtained through a cannula from a large

lymphatic of the foreleg from 5 to 50 minutes, 50 to 90 minutes, and 25 hours after injection. Urine was collected by catheterization of the bladder, 50 minutes, 105 minutes, and 5 hours after injection. Control samples of blood, lymph, and urine were taken before the experiment was started.

The concentration of Hp in blood plasma and urine was determined colorimetrically; besides, samples of plasma and lymph obtained after injection were exposed to light in the water bath at  $37.5^{\circ}$  and the oxygen consumption per cc. of substrate before and during the irradiation was compared with that of control substrate without Hp as well as of samples containing known concentrations of Hp.

The results concerning the blood plasma are given in Fig. 1, in which the colorimetric readings are plotted against time. Since the amount of Hp injected was calculated to be 1 mg. per cc. of plasma, the samples of plasma removed at various intervals were compared with a standard solution of 1 mg. of Hp in control plasma. On Fig. 1 is given the curve obtained by plotting the volume of oxygen consumed per cc. of each sample during 30 minutes exposure to light against time. The volume of oxygen consumed by control plasma without Hp during the exposure of 30 minutes duration to light of the same intensity was deducted from all values. Each point represents the average of four experiments. For comparison, the curve showing the influence of various concentrations of Hp, ranging from  $10^{-3}$  to  $10^{-\infty}$  per cc., on the oxygen consumption of control plasma during exposure to light under identical conditions is given on Fig. 1. The amount of Hp contained in the various samples of plasma removed after injection can be determined by drawing an abscissa from any point marked  $\times$  of the curve to the right until it intercepts the curve showing the influence of the concentration of Hp on photooxidation of plasma; an ordinate drawn from the point of interception down to the bottom scale gives the concentration of Hp contained in the sample.

A similar experiment was performed on a rabbit and the curve representing the volume of oxygen consumed during irradiation per cc. of plasma removed at intervals after intravenous injection of Hp is given in Fig. 2.

The method of measuring the oxygen consumption during

irradiation seems more sensitive than the colorimetric method, since samples containing 0.01 mg. of Hp per cc. consumed definitely and measurably more oxygen than such without Hp. The color of the sample of blood plasma obtained 25 hours after injection was too faint to be read colorimetrically, while the same sample consumed oxygen during irradiation comparable to control plasma containing about 0.02 mg. of Hp per cc. Therefore, the base-line

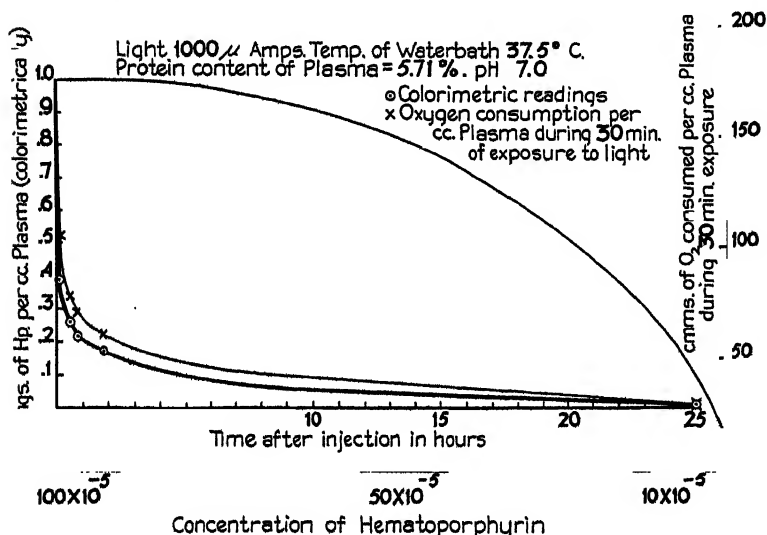


FIG. 1. Disappearance of hematoporphyrin from blood plasma after intravenous injection (50 mg. of Hp per kilo of body weight). The curve without symbols represents the influence of known concentrations of Hp on the oxygen uptake of plasma during irradiation.

representing colorimetric readings is drawn at a different level from that representing the oxygen consumption.

Colorimetric readings showed that the concentration of Hp in the blood plasma had fallen from 1 mg. per cc. at the time of injection to 0.39 mg. after 5 minutes, to 0.26 mg. after 27 minutes, to 0.22 mg. after 45 minutes, to 0.17 mg. after 95 minutes, and was too faint to be read accurately 25 hours after injection.

Measurements on the same samples on the basis of their oxygen consumption per cc. compared with the oxygen consumption of plasma containing known concentrations of Hp showed that the



plasma contained 0.24 mg. of Hp 5 minutes after injection, 0.14 mg. of Hp 27 minutes, 0.12 mg. of Hp 45 minutes, 0.093 mg. of Hp 95 minutes, and 0.023 mg. of Hp 25 hours after injection. The discrepancy between the figures obtained by the two methods is difficult to explain and it remains to be seen which of the two is the more accurate. Nevertheless the general slope of the two curves is quite similar, indicating a rapid disappearance of Hp from the blood stream after intravenous injection.

Colorimetric readings of the concentration of Hp in the urine showed that it contained 0.14 mg. of Hp per cc. 50 minutes, 0.093 mg. of Hp per cc. 105 minutes, and 0.02 mg. of Hp per cc. 5 hours after injection.

The samples of lymph collected after injection contained such a faint trace of color that they could not be read colorimetrically. When duplicate samples of lymph containing 1.03 per cent protein were irradiated under conditions identical with those described above, they consumed oxygen comparable to control lymph containing 0.009 mg. of Hp per cc. 50 minutes, 0.01 mg. of Hp per cc. 90 minutes, and 0.02 mg. of Hp per cc. 25 hours after the intravenous injection of 1 mg. of Hp per cc. of plasma.

*Appearance of Hp in Blood Stream after Intraperitoneal and Subcutaneous Injection*—50 mg. of Hp per kilo of body weight were injected intraperitoneally or subcutaneously into six rabbits and six guinea pigs and samples of blood were taken by heart puncture before and after injection at various intervals. The oxygen consumption of the plasma of these samples during the irradiation was recorded and compared with that of control plasma containing known concentrations of Hp. Several of the animals died before the end of the experiment, due to the repeated heart punctures.

The results of this study are given in Fig. 2 in which the volumes of oxygen consumed per cc. of plasma during 30 minutes exposure to light are plotted against time after injection. For comparison the curve representing the influence of various concentrations of Hp ranging from  $10^{-3}$  to  $10^{-\infty}$  on the oxygen consumption of control plasma is also given and the amount of Hp contained by the various samples of plasma removed at intervals after injection can be determined in the same way as described above. Each point represents the average of four experiments.

It can be seen that after intraperitoneal injection Hp appeared slowly in the blood stream and reached a concentration of 0.06 mg. of Hp per cc. 24 hours after injection. Because of the death of all the injected animals, data for the complete curve were not obtained.

The maximum concentration of Hp in the plasma of rabbits after subcutaneous injection was found to be 0.016 mg. of Hp per cc. 12 hours after the injection and about 0.01 mg. of Hp per

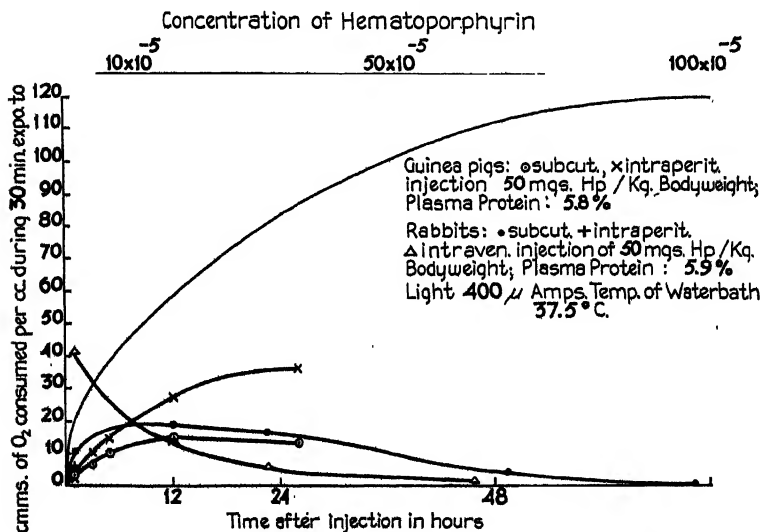


FIG. 2. Appearance of hematoporphyrin in blood plasma after subcutaneous, intraperitoneal, or intravenous injection. The curve without symbols represents the influence of known concentrations of Hp on the oxygen uptake of plasma during irradiation.

cc. in guinea pigs after the same time; no Hp could be discovered in the plasma in either animal 72 hours after injection.

The bile of the gallbladder of all animals was examined spectroscopically and was found to contain Hp after intravenous, subcutaneous, and intraperitoneal injection.

#### *The Influence of Sensitizer on Photooxidation of Tissues*

*Effect of Intravenous Injection of Hp on Oxygen Consumption of Tissues during Irradiation*—White mice of homogenous stock

of the same age and the same weight (20 gm.) were injected through the tail vein with 0.1 mg. of Hp per gm. of body weight. After a varying time ranging from 5 minutes to 6 days after the injection the animals were sacrificed; certain organs were removed and finely sliced with a sharp razor blade. The slides of tissue were suspended in Ringer's solution of pH 7 and, after being weighed, portions were placed in the respirometer bottles containing 1 cc. of Ringer's solution. The oxygen consumption was then recorded in the water bath at 37.5° before, during, and after exposure to light for a period of 30 minutes each. For comparison control tissues were studied under the same conditions and for each experimental animal a litter mate was used as control. In this manner the influence of the irradiation on the oxygen consumption of skin, liver, kidney, and brain tissue was studied. An average of five experimental animals and five controls was used for the study of each organ.

The results are given in Table I, showing the average oxygen consumption per mg. of tissue during 10 minutes of a 30 minute period. Each figure represents the mean of from eight to thirty-seven observations or an average of sixteen experiments. The column of Table I showing the difference between the readings divided by the difference of the probable errors always pertains to the figures before and during irradiation. It can be seen that the only figures of any significance at all are those showing an increase of the oxygen consumption during exposure to light of skin, 5 minutes, 30 minutes, and perhaps 1 hour after injection, and a questionably significant increase of the uptake of oxygen of liver tissue, 5 to 30 minutes after the intravenous injection of Hp.

*Influence of Concentration of Hp on Oxygen Consumption of Organ Tissues during Irradiation*—In order to compare the effect of the unknown content of Hp in the organs after intravenous injection with the effect of known concentrations of Hp on the oxygen consumption during exposure to light, tissue slices of the same organs as studied above of control animals were dipped for a few seconds in solutions of different concentrations of Hp in Ringer's solution of pH 7, rinsed with Ringer's solution, and then suspended in Ringer's solution. After certain portions were weighed out, the slices of tissue were placed in respirometer bottles containing 1 cc. of Ringer's solution and the oxygen

TABLE I  
*Oxygen Consumption of Organs of Mice after Intravenous Injection of Hematoporphyrin and Controls*  
 Average c.m.m. of O<sub>2</sub> consumed per mg. of tissue during 10 minutes of a 30 minute period.

Exposed to light, 400 microamperes									
Organ	Time after injection	Experimental animals				Controls			
		Before	During	After	(Difference) ÷ (probable error of difference)	Before	During	After	(Difference) ÷ (probable error of difference)
Skin	5 min.	2.1 ± 0.06	2.32 ± 0.05	2.1 ± 0.05	3.1	2.42 ± 0.12	2.76 ± 0.08	2.0 ± 0.1	2.4
	30 "	2.2 ± 0.1	2.77 ± 0.13	2.26 ± 0.14	3.3	2.89 ± 0.12	2.7 ± 0.1	2.42 ± 0.14	0
	1 hr.	2.45 ± 0.11	2.93 ± 0.13	2.21 ± 0.11	2.7	2.46 ± 0.05	2.6 ± 0.1	2.54 ± 0.18	1.25
	24 hrs.	2.11 ± 0.07	2.14 ± 0.06	2.09 ± 0.16	0.41	2.15 ± 0.06	2.24 ± 0.04	2.1 ± 0.1	1.25
Liver	5 min.	11.0 ± 0.29	12.7 ± 0.3	9.5 ± 0.34	2.9	12.1 ± 0.26	11.7 ± 0.25	10.5 ± 0.38	0
	15 "	10.5 ± 0.13	10.8 ± 0.11	9.2 ± 0.2	1.7	12.2 ± 0.26	12.4 ± 0.18	11.3 ± 0.24	0.64
	30 "	12.7 ± 0.2	13.2 ± 0.23	11.5 ± 0.24	1.6	11.0 ± 0.19	11.3 ± 0.28	10.1 ± 0.15	0.9
	1 hr.	11.8 ± 0.31	12.0 ± 0.29	10.4 ± 0.25	0.5	11.1 ± 0.18	10.8 ± 0.18	10.3 ± 0.28	0
	2 hrs.	11.8 ± 0.24	12.0 ± 0.3	10.2 ± 0.3	0.52	14.1 ± 0.54	13.3 ± 0.48	11.9 ± 0.56	0
	20 "	11.8 ± 0.24	11.8 ± 0.22	10.2 ± 0.27	0	13.2 ± 0.15	13.7 ± 0.39	11.4 ± 0.29	1.2
Kidney	6 days	12.8 ± 0.47	12.4 ± 0.4	11.6 ± 0.36	0	12.1 ± 0.3	12.3 ± 0.19	9.2 ± 0.19	0.57
	5 min.	30.6 ± 0.69	29.0 ± 0.35	28.0 ± 0.34	0	29.6 ± 0.08	29.0 ± 0.09	26.2 ± 0.28	0
	15 "	28.7 ± 0.43	29.7 ± 0.38	29.3 ± 0.53	1.75	31.6 ± 0.47	30.5 ± 0.83	31.7 ± 0.21	0
	30 "	32.4 ± 0.7	30.0 ± 0.53	30.0 ± 1.2	0	32.7 ± 0.43	33.1 ± 0.27	30.0 ± 0.72	0.8
Brain	1 hr.	32.3 ± 1.27	31.1 ± 0.53	30.4 ± 0.85	0	32.8 ± 0.7	32.2 ± 0.82	30.0 ± 1.2	0
	30 min.	6.4 ± 0.12	5.9 ± 0.09	5.9 ± 0.14	0	6.9 ± 0.03	7.0 ± 0.21	6.0 ± 0.18	0.47
	1 hr.	7.1 ± 0.24	7.1 ± 0.16	6.4 ± 0.13	0	7.5 ± 0.19	7.1 ± 0.15	6.2 ± 0.14	0
	2 hrs.	7.1 ± 0.16	7.3 ± 0.15	6.1 ± 0.1	0.99	7.1 ± 0.17	6.9 ± 0.21	5.9 ± 0.14	0

consumption of these tissues was then studied in the water bath before and during exposure to light. The results are given in Table II which shows the oxygen consumption per mg. of tissue during 10 minutes of a 30 minute period. Each figure represents the average of five observations.

It can be seen that the oxygen consumption of tissue slices increases rapidly during the irradiation with increasing concentrations of Hp. Similar results were obtained with slices of tissue suspended in solutions of Hp of various concentrations in Ringer's solution.

TABLE II

*Effect of Dye Concentration on Photooxidation of Tissues*

C.mm. of oxygen consumed per mg. of tissue during 10 minutes of a 30 minute period.

Organ	Before exposure	Exposed to light, 400 microamperes			
		No hematoporphyrin	Concentration of hematoporphyrin		
			$10 \times 10^{-4}$	$50 \times 10^{-4}$	$100 \times 10^{-4}$
Skin.....	2.4	2.4	4.66	10.7	13.3
Brain.....	7.2	7.1	13.3	24.3	27.3
Liver.....	12.3	12.4	21.3	31.3	35.3
Kidney.....	31.4	31.8	40.4	52.6	58.0

#### *Excretion of Hp after Intravenous Injection*

Urine, bile of the gallbladder, and the contents of the intestines of each experimental animal were examined spectroscopically. It was found that urine of the mice contained Hp sporadically; Hp was found in the bile of the gallbladder of all animals killed from 5 minutes to 24 hours after the intravenous injection of Hp. The contents of the intestines showed the presence of Hp in all animals from 15 minutes up to 24 hours after the injection and was sometimes seen as early as 5 minutes after injection.

#### DISCUSSION

The rapid disappearance of Hp from the blood stream and its scarcity in the tissues after intravenous introduction demonstrate the efficiency with which the body is able to dispose of this pathological material. Practically all of the injected Hp is taken up by

the liver and excreted through the biliary tract into the intestinal canal; sometimes it is partly eliminated by the kidneys. On the basis of these experiments the effect of irradiation almost 2 months after intravenous injection of Hp, as reported by Meyer-Betz (2), is difficult to understand.

If Hp is introduced intraperitoneally or subcutaneously, it appears in the blood stream soon after injection, reaches a certain maximum concentration, and gradually disappears when the depot is exhausted; from the blood stream it is again taken up by the liver and excreted into the intestinal canal by way of the biliary tract. The concentration of Hp present in the blood stream after parenteral introduction then depends on the amount of Hp injected, the method of administration, the time after injection, the rapidity of absorption, and the efficiency of elimination. Since photosensitization takes place only when sensitizer and acceptor are present and in contact with one another during irradiation, the effect of irradiation of an animal injected with Hp would then depend on the same factors, all other conditions being equal.

From previous studies (1) showing that the proteins of body fluids are oxidized during irradiation in the presence of a sensitizer, it can be assumed that proteins of cells are the acceptors in photooxidation of tissues; this has actually been demonstrated for muscle tissue by Kosman and Lillie (3). It is not known what proteins of cells furnish the acceptors; different tissues apparently contain different quantities of suitable acceptors, because the oxygen consumption during irradiation varies with tissues of different organs even if all other factors are identical.

The increase of oxygen uptake during exposure to light in the presence of a sensitizer is due to the oxidation of acceptors and is different from ordinary cell respiration in that it does not depend upon cellular structure, is not inhibited by hydrocyanic acid, and is not influenced materially by temperature (4). The findings of Medvédéva (5) that photodynamic substances (chlorophyll and fluorescein) cause a greater increase of tissue metabolism in the dark than during irradiation are contrary to those of most other observers. The gaseous metabolism of living animals sensitized with eosin and exposed to light and the influence of adrenalin on normal and sensitized animals during irradiation and in the dark were studied by Gatsaniouk (6). He also reports a greater oxygen

uptake of sensitized animals kept in the dark than of those exposed to light.

The studies on the photooxidation of tissue slices of internal organs after intravenous injection of Hp indicate that the concentration of sensitizer in these organs is negligible; it may therefore be concluded that the rôle which these organs play in photosensitization of living animals is minimal. In addition to the low concentration of sensitizer within these organs, much of the light is absorbed by the surface layers of the animal, so that the photodynamic effect on these organs is rather small.

The intravenous introduction of Hp seemed the best way to study the effect of sensitizer on different organs because of its greater concentration in the blood stream—at least soon after injection—which level is never reached after intraperitoneal or subcutaneous application. Since Hp injected parenterally is taken up from the blood stream by the liver, its concentration in this organ during the 1st hour after intravenous injection must necessarily be greater than that at any time after intraperitoneal or subcutaneous injection. The fact that its actual concentration in this organ under these optimal conditions is so low as to be almost not measurable strongly supports the idea that the effect of photosensitization of living animals does not depend on the photooxidation of tissues of internal organs.

The oxygen consumption during irradiation of skin after intravenous injection of Hp is not greatly increased, indicating that little sensitizer is present; the small amount which is demonstrable is probably contained in the blood of the skin capillaries. No evidence of any storage of Hp in the skin could be demonstrated 24 hours after injection. Nevertheless it seems safe to assume that the skin and its capillaries play the most important rôle in the photosensitization of living animals: light can easily penetrate through the epidermis and reach the capillary blood stream containing a measurably effective amount of Hp within a certain time after the parenteral injection of the sensitizer. The proteins of the plasma as well as those of the tissues which are in direct contact with the sensitizer are oxidized during irradiation; oxygen is easily available within the circulation and it, as well as the acceptors, is constantly replenished.

According to Ellinger (7) the number of open capillaries in the

human skin of certain areas is about 35 per sq. mm.; if this holds true for animals, the surface of the capillary bed reached during irradiation is considerable.

The general effects of photosensitization of the living animal would then depend on the size of the surface area subjected to irradiation and would be due to the consequences of this primarily local damage.

#### SUMMARY

1. The concentration of the hematoporphyrin in the blood stream after intravenous introduction decreases rapidly within 1 hour and is barely measurable 24 hours after injection.

2. After intraperitoneal or subcutaneous introduction the hematoporphyrin appears soon in the blood stream, reaches a maximum concentration in from 12 to about 24 hours, and then gradually disappears from the circulation.

3. Most of the hematoporphyrin circulating in the blood stream is taken up by the liver and is eliminated into the intestinal canal through the biliary tract; a small portion may sometimes be excreted through the kidneys into the urine.

4. The concentration of hematoporphyrin in the internal organs after its intravenous introduction into living animals is negligible.

5. Tissues of organs can be oxidized during irradiation in the presence of a sensitizer.

6. The effect of photosensitization of living animals is due to the oxidation of acceptors of the plasma within the capillary circulation of irradiated skin and tissues of skin in contact with the sensitizer.

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## ACTIVATION OF ENZYMES

### IV. THE JACK BEAN ARGININOLYTIC ENZYME

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In connection with a study of the rôle of certain metal ions as "activators" of liver arginase, Hellerman and Perkins presented evidence (1) for the existence of an enzyme in the jack bean which accelerates the hydrolysis of *d*-arginine. This catalyst at present may be referred to as jack bean arginase. Its activity, like that of liver arginase, was found to be enhanced significantly under well defined conditions by cobaltous ion or by certain other heavy metal ions. The decomposition of *d*-arginine in the presence of the activated enzyme and of accompanying urease yielded, after a sufficient reaction time, almost 2 equivalents of ammonia.

The description of the initial experiments ((1) pp. 189, 190) brought out that relatively *crude* preparations of jack bean urease, including crude crystalline urease, had been observed to possess argininolytic properties. Highly recrystallized urease (*e.g.*, thrice recrystallized or more) was not used. There is no present reason to assign any catalytic activity other than the ureolytic to the highly recrystallized product, the identity of which as a homogeneous protein is suggested by several lines of evidence. Therefore, the reference to crystalline protein urease in the paper by Hellerman and Perkins was not to have been construed as implying that the urease molecule itself, as represented by Sumner's highly recrystallized preparation, may in the presence of cobaltous ion, etc., assume argininolytic properties. Actually, recrystalliza-

\* This paper is based in part upon a section of a thesis submitted by C. Chester Stock in partial fulfillment of the requirements for the degree of Doctor of Philosophy, the Johns Hopkins University. See following paper (11).

tions of urease appear to eliminate progressively the arginase activity<sup>1</sup> (cf. (2)). The crude crystallized preparations provided a convenient source of jack bean arginase, activatable by appropriate heavy metal ions. The similarities in certain characteristics of the jack bean and liver argininolytic enzymes offer interesting suggestions concerning the nature of the arginine-arginase reaction.

The evidence underlying the working hypothesis that *liver* arginase may consist of a metallo complex has been cited elsewhere (1, 3). It may be considered that a more or less separable metallic component is bound, presumably by coordinate bonds, to "donor" groups of a non-metallic part, probably of protein nature. It has been indicated further that the substitution of a constituent of the jack bean for the donor component of liver arginase and of such an ion as cobaltous for the metallic portion, in the enzymatic hydrolysis of *D*-arginine, suggests in a sense the construction of an artificial arginase (3). If, moreover, metallo-enzyme be assumed to have combined with *D*-arginine in the formation of a dissociable enzyme-substrate complex, a primary factor in the control of stability of the resulting complex molecule might reside in the coordinative linking of the enzyme's metal ion to the substrate's  $\alpha$ -amino group. Such a process virtually would involve a competition between enzyme-metal ion and hydrogen ion for the substrate's  $\alpha$ -amino group in the pH range in which arginase is active. This is consistent with the following. From the magnitude of arginine's several dissociation constants (4) it follows that the zwitter ion of isoelectric arginine,  $+[H_2N=C(NH_2)-N(H)]-(CH_2)_3-C(H)(NH_2)-COO^-$ , involves essentially the guanidinium cation and the carboxyl anion. In a titration of arginine from its isoelectric point to a region of lowered pH (e.g., pH 6.5), there is concerned chiefly the conversion of the  $\alpha$ -amino group to the  $\alpha$ -ammonium configuration ( $-NH_2 \rightarrow -NH_3^+$ ). The work of Hunter (5) and of Edlbacher (6, 7) and their coworkers with pH-activity curves shows that liver arginase functions in a wide pH range (roughly pH 5 to 11). The liver preparation used by Hellerman and Perkins appears to contain material characteristic of native arginase, together with some inactive enzyme which assumes argininolytic activity in the

<sup>1</sup> Perkins, M. E., unpublished experiments.

presence of certain salts. If our concepts relative to arginase have any validity, it must be anticipated that the addition of activating metal ion to arginine-arginase mixtures such as we have used should alter significantly the pH-activity curve. The argument may be extended *mutatis mutandis* to the behavior of jack bean arginase.

This paper records exploratory observations upon the relation between activity and pH for jack bean arginase and upon modifications introduced by the presence of cobaltous, nickelous, or manganous salts, while the following paper gives similar data for the liver enzyme. There is presented also a brief account of the kinetics of *d*-arginine hydrolysis in the presence of jack bean arginase and cobaltous ion. In addition, there are included some experiments which illustrate the distortion of the titration curve of arginine in the presence of cobaltous, nickelous, or manganous ions and show in a preliminary way the relative abilities of these ions *alone* to combine with the substrate amino acid.

#### EXPERIMENTAL

*Activity-pH Curves of Jack Bean Argininolytic Enzyme* — The characteristics have been determined on the basis of the extent of hydrolysis of *d*-arginine in a given time, rather than by the ideal method of comparing the initial reaction velocities. The procedure was considered admissible, since for comparative purposes only the main characteristics of the several curves were of interest. Pertinent is the demonstration by Hunter and Morrell, working with certain preparations of liver arginase, that curves so obtained are roughly similar to those given by a more rigorous method.

Determinations were carried out as follows: To a solution (contained in a 25 ml. volumetric flask of resistant glass) composed of 10 ml. of buffer (see below), 46 mg. of *d*-arginine hydrochloride, and appropriate heavy metal salt were added caprylic alcohol (2 drops), 5 ml. of a solution containing 0.1 gm. of crude jack bean preparation (8) or crude crystalline (9) urease of comparable argininolytic activity, and redistilled water to make 20 ml. The mixture was kept for 2½ hours at 30°, after which there was added 1 ml. of 0.25 *M* sulfuric acid and the solution was heated 10 minutes at 100°; it was cooled and diluted to 25 ml. After filtration, ammonia in 10 ml. was determined by the usual aeration method

(cf. (1)) and estimated in terms of ml. of 0.0200 N HCl neutralized. Appropriate controls were carried out upon reagents and the heat-denatured enzyme. Water redistilled in glass was used in all enzyme work.

When activating metal salt was employed, there were present 3 mg. of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 5 mg. of  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , or 3 mg. of  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ .

The buffers used were acetate, phosphate, and glycine, respectively, for the ranges of pH 4.5 to 5.5, 5.7 to 7.8, and 8.2 to 12.6. The phosphate buffers (10) were used so that the *ionic strength* in the reaction mixture was 1. The glycine buffers were used with approximately the same ionic strength, while acetate buffers were employed in a final concentration of 1.0 M with respect to total acetate. The pH of each buffer at the proper dilution was determined by means of a hydrogen electrode. Since the buffer salts may in varying degree display characteristic specific effects, some care must be taken in the comparison of results obtained with different kinds of buffers. The more significant changes resulting from the presence of the activating metal ions occur in the range covered by the phosphate buffers; primary consideration is to be given to the data obtained in this range.

Fig. 1 gives the mean activity-pH curve for jack bean arginase and records, in addition, the data obtained when there was present cobaltous, nickelous, and manganous salt, respectively. Each curve is based upon a number of independent determinations of the relative activities through a range of pH from 4.5 to 12.6 at intervals of 0.05 to 0.50 pH unit, the magnitude of the intervals depending upon the degree of change of slope of the curve (cf. (11)).

*Kinetics of d-Arginine Hydrolysis in Presence of Jack Bean Arginase and Cobaltous Ions* — A suitable rate study promised to throw light upon the action of the argininolytic enzyme and upon the part played by *urease* in the over-all process. The results will be discussed in another section.

After considerable preliminary work, there were found the conditions under which consistent, reproducible results were obtainable. Concentrations of certain reagents greater than those used in earlier work were found essential. Especially important was the use of more concentrated buffers, since under

the conditions (pH 7.5) a small change in pH affects appreciably the activity of cobalt-enzyme (*cf.* Fig. 1). The data reported here are from one of five similar runs, all of which yielded results which fit the simple first order relation. The values of the several average reaction constants obtained differed somewhat owing to variations in the enzyme content of the preparations used.

Under the conditions of the present observations the urease contained in the jack bean preparations used has been found to effect in less than 1 minute the hydrolysis of a quantity of urea

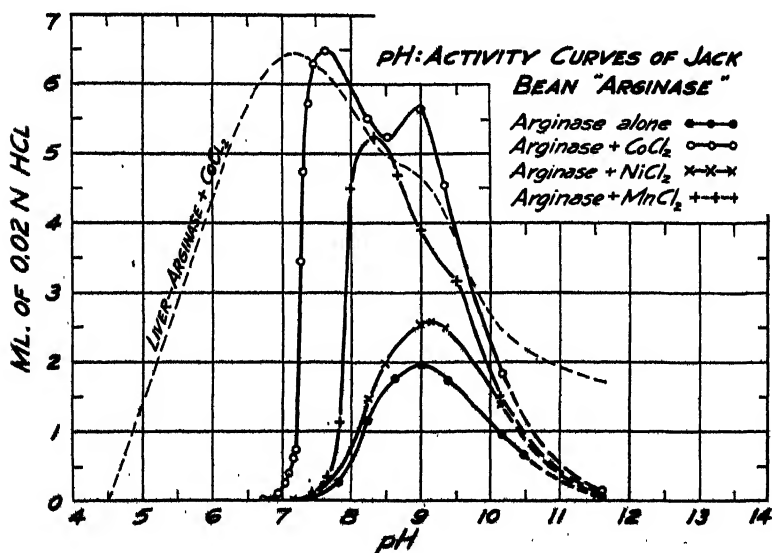


FIG. 1

equivalent to the total amount of the *d*-arginine originally present in the reaction mixtures. This is far more urea than could have been present at any time during hydrolyses of *d*-arginine in the presence of the jack bean enzymes under the conditions described (*see* (9)).

**Procedure**—Solutions of *d*-arginine hydrochloride and jack bean preparation with cobaltous ion and buffer were mixed and kept at 30.0°. The time of mixing was noted and at definite intervals (*t* in Fig. 2) a 20 ml. sample was withdrawn from the reaction mixture. This was added to 1 ml. of hydrochloric acid

(1 M) in a 25 ml. volumetric flask, kept at 100° for 10 minutes and cooled. After the addition of water to the mark and subsequent filtration, 10 ml. samples were removed and aerated in the usual

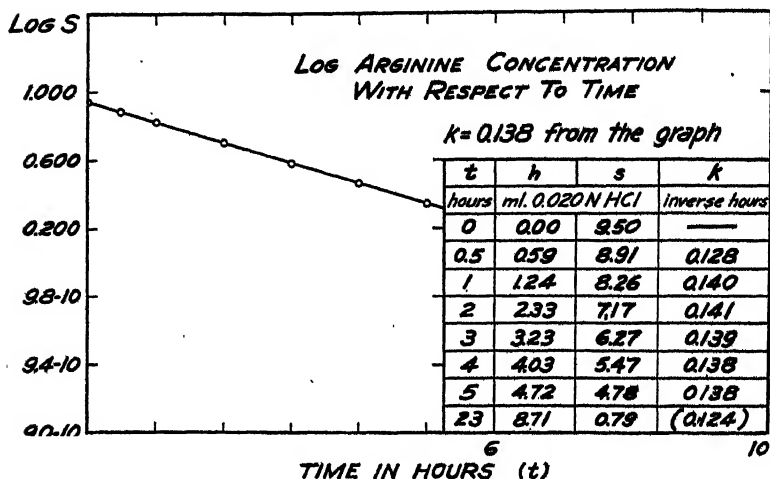


FIG. 2. *d*-Arginine hydrolysis in the presence of jack bean arginase and cobaltous ion. The reaction mixture, total volume 160 ml., contained *d*-arginine hydrochloride 0.400 gm.,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.016 gm., phosphate buffer 1 M (pH 7.5) 80 ml., crude crystalline jack bean urease 8 ml., redistilled water to volume, a few drops of caprylic alcohol. The enzyme solution (8 ml.) contained crude crystalline urease derived from 32 gm. of jack bean meal (of an Arkansas crop purchased on the market). The rate of disappearance of the substrate under the conditions may be formulated as  $-ds/dt = ks$ , from which  $k = (2.303/t) \times (\log a \text{ minus } \log s)$ , where  $k$  = the "specific reaction" constant,  $s$  = moles of substrate (*d*-arginine) present at time  $t$ , and  $a$  = the initial *d*-arginine concentration. For  $a$  may be used the calculated ml. of 0.0200 N HCl equivalent to the ammonia corresponding to complete hydrolysis of the *d*-arginine in 0.4 of a 20 ml. sample of reaction mixture (see the text); on the basis of 2 equivalents of  $\text{NH}_3$ ,  $a = 9.50$ . For  $s$  is used the value obtained by subtracting from  $a$  the ml. of 0.0200 N HCl required to neutralize the  $\text{NH}_3$  formed at time  $t$  ( $h$  column). (The values listed for  $h$  have been corrected for the control on reagents and enzyme in absence of substrate; control = 0.10 ml. of 0.0200 N HCl.)

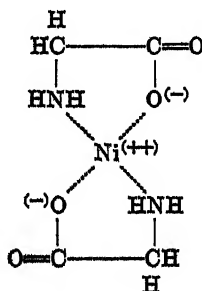
manner for the estimation of ammonia. Further details and the results are given in Fig. 2.

*Potentiometric Titrations; Distortion of Titration Curve of d-*

*Arginine in Presence of  $Ni^{++}$ ,  $Co^{++}$ , or  $Mn^{++}$*  — Data presented in this and the following paper disclose that effective heavy metal ions not only "activate" the argininolytic enzymes, but, in addition, induce shifts in their activity-pH curves. As a preliminary, it seemed appropriate to inquire to what extent the modifications are related to the ability of the substrate, *d*-arginine, to form complexes with such ions. An exploratory study has been made with cobaltous, nickelous, and manganous salts.

From the introductory paragraphs it might be inferred that an extension of the range of enzyme activity into a more acid region in the presence of cobaltous ion, for example, is to be correlated with the ability of cobaltous (or more pertinently the enzyme-cobaltous) ion to compete effectively in that region with hydrogen ion for a donor group such as the  $\alpha$ -amino group. In addition, the precipitation of a metallous hydroxide would play its own part in the modification of the activity-pH relations.

In our experience the actual isolation of nickelous or cobaltous complexes of arginine is not so simple a matter as the preparation of corresponding complexes derived from glycine or alanine. The latter were first studied by Ley (12). Nickelous glycine, neutral in character, may be designated by the accompanying formula. Although the situation is probably somewhat similar



for *d*-arginine, the presence of guanidinium groupings may influence the physical properties of the complexes (here, complex ions) in the direction of increased solubility, etc.

Early observation that complex formation with amino acids hinders the alkaline precipitation of metal ions has been elaborated by so called anomalous titrations. Such titrations have been



made upon mixtures of glycine and  $Mg^{++}$  by Zörkendörfer (13), upon hydroxy acids and  $Fe^{+++}$  or  $Cu^{++}$  by Smythe (14), and upon hydroxy acids (and arginine) and  $Mn^{++}$  by Main and Schmidt (15). In these studies there have been disclosed interesting shifts in the titration curves of various compounds in the presence of metal ions.

In order to obtain data which might permit an approximation of the order of magnitude of the dissociation constants of metallo-arginine complexes, there have been carried out in this work a number of potentiometric titrations. With sodium hydroxide, solutions of nickelous, cobaltous, and manganous chlorides were titrated for the determination of the ranges of pH in which the ions are precipitated as "hydroxides" (cf. (16, 17)). In addition, *D*-arginine hydrochloride has been titrated in the presence of each of these salts. To the metal salt solutions to be titrated was added sodium chloride to its concentration in the arginine-metal ion titration mixtures at the point of hydroxide precipitation (i.e., about equivalent to the sodium hydroxide used). In the zones of precipitation there were encountered drifts in the hydrogen ion activity to more acid values, which in some cases persisted for several hours before the change in pH during 10 minutes was 0.01 pH unit or less; therefore, care was taken to determine carefully several points after the first definite precipitation of a metalous hydroxide was observed. Titrations were attempted at different times with both glass and hydrogen electrodes, the working half-cell being a saturated calomel electrode. The glass electrode used permitted the measurement of changes in pH of 0.005 pH unit. It was calibrated on each day of use by measurement of six or seven appropriate buffers, the pH of which had been determined with a hydrogen electrode. In the range of pH 2.0 to 8.0, there were obtained with the glass electrode the theoretical values of  $\Delta E/\Delta pH$ ; for the more alkaline range there were used calibration data in order to convert measured potentials to pH units. For the measurements with glass electrodes, the temperature was controlled in an air bath at  $30.0^\circ \pm 0.03^\circ$ . Purified nitrogen was used to stir the solutions and exclude air from the titration vessel, which was similar to that described by Clark (16).

Discrepancies were noted in the determination of the pH zones of precipitation of the metal hydroxides both in the presence and

absence of arginine when the results obtained respectively with the glass and hydrogen electrodes were compared. The values obtained with the glass electrode are considered more reliable, for apparently the glass electrode is not affected by the hydroxides nor are the latter altered by the electrode. Nevertheless, the general results with both electrodes are similar qualitatively. So far as titrations of amino acid alone are concerned, they agree quantitatively. The heavy metal salts used were high grade commercial products; they were analyzed and upon the analyses were based the concentrations of metal ions used in the titrations. By precipitation of nickelous dimethylglyoxime, the  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  used was found to contain the theoretical amount of nickel. The  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  was found, by potentiometric titration with potassium ferricyanide (18), to contain 97.1 per cent of the theoretical cobalt content. In the  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , the manganese content was found by the use of sodium bismuthate to be 99.6 per cent of the theoretical amount. The purity of *d*-arginine and other organic compounds titrated was established by suitable analyses (total and  $\alpha$ -amino N) and by the correspondence of the theoretical and observed end-points in appropriate titrations.

In addition to *d*-arginine, a number of other compounds were titrated in the presence and in the absence of  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ , and  $\text{Mn}^{++}$ . Such compounds were glycine,  $\delta$ -guanidinovaleric acid, and *d*-ornithine dihydrochloride. In most of these cases, the detailed characteristics are of little concern to the central topic of the present communications and are not presented here. They will be considered as occasion demands.<sup>2</sup>

Fig. 3 presents the titration curves (with the glass electrode) of *d*-arginine in the presence of and in the absence of several heavy metal salts. With the detail of the foregoing text the figure is self-explanatory. By way of illustration of the titrations of metal salts<sup>2</sup> alone some data are presented from the titrations of nickelous chloride in the absence of amino acid. Table I gives illustrative results.

An analysis of the potentiometric titrations might permit some decisions regarding both the composition of the *d*-arginine-metallic

<sup>2</sup> The details of all titrations are recorded in the dissertation of C. Chester Stock, on file in the library of the Johns Hopkins University.

complexes and the dissociation constants thereof. However, there would necessarily have to enter certain assumptions, the verification of which would require more extensive investigation. Since the immediate objective is met by a semiquantitative analysis of the data at hand, we prefer to leave the more rigid analysis to the future and shall proceed with the argument within restricted premises. On the basis of available collateral information concerning the nature of such nickelous complexes of the simpler amino acids as have been isolated (12) and of the nickelous, cobaltous, and manganous ammonium ions, it might be supposed that arginine forms with such metal ions an equilibrium mixture of complexes in which the form 2 arginine-1 metal ion quantitatively is an important component. Borsook and Thimann<sup>3</sup> (19) came to a similar conclusion in their studies of cupric glycine. If it be assumed that a complex ion of this type is most characteristic for a divalent metal ion-arginine complex *in the pH region where precipitation of the metal hydroxide is imminent*, the dissociation constant  $K_c$  of the postulated complex  $((\text{RNH}_2)_2\text{Me})^{++}$  may be calculated with the aid of the expression

$$\frac{[\text{Me}^{++}][\text{RNH}_2]^2}{[(\text{RNH}_2)_2\text{Me}]^{++}} = K_c$$

Substitutions are made with the use of the solubility product relation  $K_s = [\text{Me}^{++}][\text{OH}^-]^2$  for a given metal hydroxide and the equilibrium relation for the dissociation of arginine's  $\alpha$ -ammonium group ( $K'_2$  under the conditions (see text) is taken as  $1.29 \times 10^{-9}$ ). Illustrative results for such calculations, based upon the data obtained in this work, are as follows:<sup>3</sup>

Metal hydroxide	$K_s$	Postulated complex	$K_c$
Nickelous.....	$4.5 \times 10^{-15}$	$(\text{Ni (arginine)}_2)^{++}$	$6 \times 10^{-11}$
Cobaltous.....	$6.5 \times 10^{-15}$	$(\text{Co (arginine)}_2)^{++}$	$2 \times 10^{-8}$
Manganous.....	$1.3 \times 10^{-13}$	$(\text{Mn (arginine)}_2)^{++}$	$7 \times 10^{-5}$

<sup>3</sup> Approximations of the dissociation constants of nickelous-, cobaltous-, and manganous-arginine, presented in a preliminary report (20), were based upon data obtained with the use of the hydrogen electrode. For reasons already cited, they are considered less reliable than the values given here.

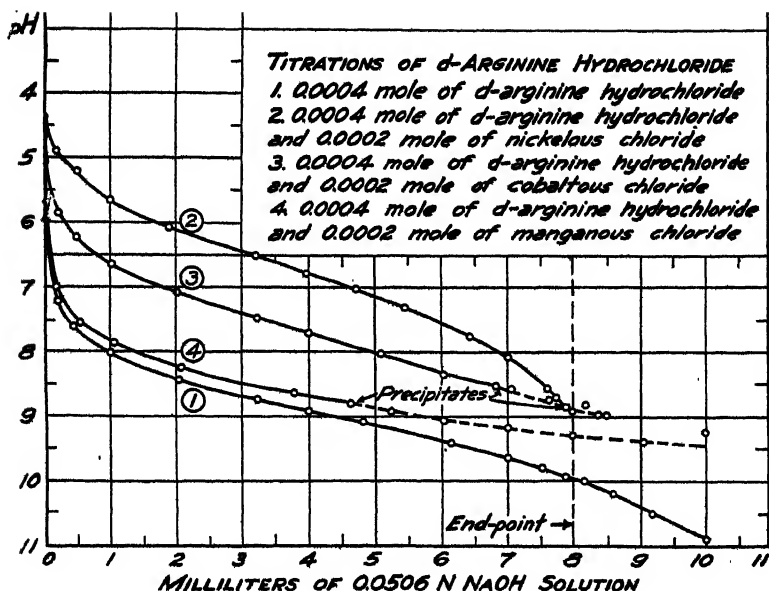


Fig. 3

TABLE I

*Titrations of Nickelous Chloride with Sodium Hydroxide*

Solution A. 40 ml. containing 0.0002 mole of nickelous chloride and 0.00032 mole of sodium chloride. (The latter salt was added in an amount equal to that formed during the titration of arginine hydrochloride and nickelous chloride to the point of precipitation of nickelous hydroxide.)

Solution B. 50.4 ml. containing 0.0002 mole of nickelous chloride and 0.0004 mole of sodium chloride.

A glass electrode was used; temperature,  $30.0^\circ \pm 0.08^\circ$ .

Solution A		Solution B	
0.0506 N NaOH	pH	0.0500 N NaOH	pH
ml.		ml.	
0.00	6.20	0.00	5.45
0.04	7.82	0.03	6.45
0.08	7.90*	0.06	6.89
0.12	7.93†	0.17	7.90*
	7.75‡	0.23	7.97
		0.45	8.00
0.82	7.92†	0.75	8.00
	7.75‡	0.97	8.00

\* Precipitation of metal hydroxide.

† Initial pH after addition of NaOH.

The determination of the hydrogen ion concentration at the point where a metal hydroxide just begins to precipitate when an amino acid-heavy metal salt mixture is titrated with sodium hydroxide rests upon the assumption that the solutions are in true equilibrium with the solid phase which, for present purposes, is considered to consist essentially of the species  $\text{Me}(\text{OH})_3$ . This introduces a rather important source of uncertainty. The observations upon which the illustrative results are based were found to be fairly reproducible. It may be noted that an error in the determination of pH at the precipitation point amounting to 0.05 to 0.1 pH unit would change the values of  $K_s$  within a range of  $1 \times 10^4$ . The experimental difficulties and the assumptions underlying the calculations of the dissociation constants,  $K_s$ , strictly limit their applicability. However, the numerical values, presented for comparative purposes, suffice as tentative approximations. A more rigorous analysis should furnish a more general and useful evaluation.

#### DISCUSSION

In Fig. 1 is depicted the effect of certain activating heavy metal ions upon *d*-arginine hydrolysis in the presence of the jack bean argininolytic enzyme with buffers of widely varying pH values but of approximately constant ionic strength. Further elaboration of such data did not seem advisable with the enzyme preparations available. The activity-pH curves show characteristic differences. Manifest is the enhancement of catalytic activity in the presence of cobaltous and of manganous ion. Of particular interest is the ability of the cobaltous ion to effect an extension of the range of enzyme activity into a pH region considerably less alkaline than that observed for the enzyme without added heavy metal salt. The range of optimal activity is shifted from the region of pH 9 to about pH 7.7. Even more striking is the steep ascent to its first inflection of the curve representing cobaltous-activated enzyme; the rise to a maximum is within a pH unit or less. This is in marked contrast to the behavior of liver arginase (see Fig. 1 and (11)). For the latter enzyme the inception of cobaltous activation is at pH 4.5, the activity being maximal at pH 7.5. On the other hand, jack bean enzyme with  $\text{Co}^{++}$  displays negligible activity below pH 6.7, and maximal at pH 7.7. Another

point of difference is seen in the behavior of the enzymes with nickelous ion; with nickel salts, under the conditions used, the jack bean enzyme is not efficiently activated. These and other differences constitute strong evidence of the non-identity of the two catalysts as chemical individuals.

From the observations of negligible jack bean argininolytic activity below pH 6.7 (with or without added metal ion) arises a consideration of some importance in analytical work. For example, an adjustment of the hydrogen ion concentration to pH 6.0 in a reaction mixture containing jack bean enzymes can be used to eliminate the argininolytic activity; under the conditions the ureolytic principle remains sufficiently active for analytical purposes.

The activity-pH curve for cobaltous-enzyme displays an *apparent* subsidiary optimum around pH 9, which is approximately the region of characteristic inflection for the "native" jack bean enzyme. There is a *suggestion* of a second optimum also in the curve for manganous-enzyme. Elaboration of the argument that each of these curves reflects the properties of a mixture of enzymes differing only in their metallic components would be gratuitous at present. Similar behavior is exhibited by liver arginase (11).

Although it has been found convenient to visualize the argininolytic enzymes as metallic complexes and this conception has aided experimentation, it must be recalled that there is as yet little direct evidence of their chemical nature. Moreover, there is no anticipation that the structure of any coordination complex elaborated by combination of a metallo-enzyme and its substrate, or the stability of the resulting molecule and its other attributes, would be closely comparable with the corresponding characteristics of a simple amino acid-metal complex such as nickelous-arginine or nickelous-glycine. Nevertheless, it was deemed advisable, for reasons discussed earlier in this paper and in order to gain additional information regarding the general behavior with amino acids of the activating ions, to ascertain the relative abilities of certain of these ions to combine with *D*-arginine. The measurements have shown that for the salts used specifically in the activity-pH studies, the respective ions in the order of stability of their complexes with *D*-arginine are  $\text{Ni}^{++} > \text{Co}^{++} > \text{Mn}^{++}$ . A similar relationship also was found<sup>2</sup> to obtain, qualitatively,

for the corresponding complexes of glycine. The presence of heavy metal ions used in these titrations has thus the effect of displacing the titration curves of the  $\alpha$ -amino groups of either glycine or *d*-arginine to a more acid region. In titrations designed to indicate the shift in pH with respect to variation in the ratio of concentrations of cobaltous (or nickelous) ion to *d*-arginine, it was observed that the greater shifts occurred when a comparatively small proportion of metal ion had been added; for example, there was little further shift in the titration curve of *d*-arginine's  $\alpha$ -amino group when there had been added heavy metal ion in excess of an amount equivalent to the *d*-arginine present. While these titrations have yielded no final information concerning the composition of the complexes, they are of interest in connection with some observations of Levy (21) dealing with titrations of *d*-arginine in the presence of formaldehyde. Of the latter reagent, an amount greatly in excess of the concentration of *d*-arginine was required to produce the largest shifts in the titration curves.

Of considerable importance to this investigation is the question whether a relationship exists in the variations to be noted in the activity-pH curves and the relative ability of effective activating ions, in general, to form coordination compounds; *e.g.*, with *d*-arginine. In considering this question it must be emphasized that the conditions under which the two sets of data were obtained are conspicuously different. For example, heavy buffering is, of necessity, required in the pH-activity studies. Furthermore, the postulated non-metallic donor component<sup>4</sup> of the enzyme would play a rôle in the shaping of the pH-activity curves, not alone through variation of its stability with changing pH (*cf.* (22)) but further through changes in ionic species involving groups more or less associated with its activity. Such effects may also account in part for the major differences in the corresponding pH-activity curves for liver arginase and the jack bean enzyme. The ability of effective heavy metal ions to cause a shift in the enzyme's pH-activity curve is less apparent in jack bean arginase than in liver arginase (11), because the pH range of activity is in general less broad in the former case. Nevertheless, it seems clear that

<sup>4</sup> *Metallo-enzyme* would represent a kind of "holoenzyme"; donor component of arginase, the corresponding "apoenzyme" (*cf.* (23)).

under the conditions cobaltous ion has widened the range of activity of the jack bean enzyme also, effecting a definite *shift* of the ascending branch of the activity curve into a less alkaline region. A similar extension evidently is not associated with the action of the manganous ion, an almost equally potent activator. It is tempting to correlate these observations with the findings concerning the stability of *d*-arginine-metallic complexes; specifically, that the stability of certain *d*-arginine-cobaltous complexes is appreciably greater than of supposedly comparable *d*-arginine-manganous complexes. Is the shift of the activity curve in the presence of the cobaltous ion to be attributed, in a measure, to the ability of the metal ion, through coordination with  $\alpha$ -amino groups (and carboxyl anions) to "withdraw" the substrate, *d*-arginine, in the region of activity of the corresponding metallo-enzyme?

There remain to be considered the results of experiments dealing with the kinetics of hydrolysis of *d*-arginine. When the catalyst is jack bean arginase with a cobaltous salt, the process, under the conditions, is pseudomonomolecular. This is comparable with our previous observations with liver arginase. However, in the present instance urea, a product of the initial hydrolysis, does not accumulate, since urease is a constituent of the enzyme preparation used. In an earlier report ((1) p. 189) the question was raised whether the argininolytic effect here might be attributable essentially to the efficient removal of the urea formed in an initial hydrolysis of a metal-activated complex of *d*-arginine. A consideration of the relative rates of hydrolysis in the involved reactions has permitted a negative answer. In addition, as will be shown elsewhere, the *non-enzymatic* (hydroxyl ion-catalyzed) hydrolysis of *d*-arginine to urea and ornithine is very slow and is not hastened in appropriate regions of pH by the presence of small amounts of metal ions which can activate the argininolytic enzymes. The results from rate measurements are consistent with the observations referred to in the introductory section respecting the negligible effect upon *d*-arginine of cobaltous salts with highly recrystallized urease. The argininolytic action is evidently independent of the ureolytic.

In general, the results of this investigation, while suggesting



the non-identity of liver arginase and the jack bean argininolytic enzyme,<sup>5</sup> at the same time emphasize a similarity in the mode of their participation in *d*-arginine hydrolysis. For the two enzymes there has been indicated also a similar specificity (11).

#### SUMMARY

1. For exploratory purposes there have been evaluated under a severely restricted set of conditions activity-pH curves for the jack bean argininolytic enzyme. These curves depict characteristic changes induced by several effective activating heavy metal ions. The data have suggested a useful application in the estimation of urea in the presence of *d*-arginine and in the evaluation of liver arginase activity.

2. By means of appropriate potentiometric titrations there was ascertained the relative stability of complexes formed by *d*-arginine with nickelous, cobaltous, and manganous ions. The bearing of the data upon certain characteristics of the pH-activity curves has been considered.

3. Studies of the kinetics of *d*-arginine hydrolysis in the presence of the jack bean enzyme and cobaltous ion at pH 7.5 have disclosed that the process, similar to hydrolysis catalyzed by liver arginase, is pseudomonomolecular. The argininolytic activity of the jack bean depends in no observable way upon urease as such.

4. There have been pointed out certain differences in the characteristics of liver arginase and the jack bean enzyme as well as the apparent similarity in the mode of their "activation" and of their action. The results appear to have significance for the elucidation of the rôle of metal ions in the enzymatic hydrolysis of *d*-arginine.

<sup>5</sup> Iwabuchi has reported (24) failure to confirm the argininolytic action of jack bean preparations. Since he does not present his data, his work cannot be evaluated. His experiments may have been conducted in ranges of pH unfavorable to the enzyme's activity or his preparations may have been poor in the argininolytic factor. Sumner and Dounce (2) have confirmed the observations (1) relative to the arginase-like property of certain jack bean preparations with cobalt salts. It is important to note that whole jack bean meal contains a hydrolyzable substrate upon which an enzyme, also contained in the meal, may act under suitable conditions, especially in the presence of activating heavy metal salts. It is conceivable that the substrate is canavanine and that the canavanase is identical with the enzyme, termed in this paper, "jack bean arginase" (cf. (11)).

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## ACTIVATION OF ENZYMES\*

### V. THE SPECIFICITY OF ARGINASE AND THE NON-ENZYMATIC HYDROLYSIS OF GUANIDINO COMPOUNDS. ACTIVATING METAL IONS AND LIVER ARGINASE

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More recent studies of the properties of arginase have stressed the probable significance of metal ion chemistry for the mechanism of its action (1). Previous reports (2, 3) from this laboratory have dealt with the inhibition of activity of liver arginase by a variety of reagents and the restoration of activity by certain heavy metal ions (especially  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ , and  $\text{Mn}^{++}$ ), with the existence of an argininolytic enzyme in the jack bean which also is remarkably activated in the presence of certain of these ions, and with evidence pointing to a possible rôle of metal-coordinative processes in the enzymatic hydrolysis of *d*-arginine.

From potentiometric titrations of *d*-arginine in the presence of heavy metal ions (3) it is clear that the  $\alpha$ -amino group plays an important rôle in the formation of metallo complexes derived from this amino acid. In this respect arginine as compared with simpler amino acids (*e.g.*, glycine) displays no essential peculiarity. This raises a question with reference to the necessity for the  $\alpha$ -amino substituent in the substrate upon which the argininolytic enzymes act. Insufficient information upon this point has been afforded by the already extensive studies upon the specificity of arginase.

\* For related studies dealing with certain adjacent fields of biological catalysis and not included in the numbered series, see (1) and *Am. J. Physiol.*, 120, 522 (1937).

† This paper is based upon a section of a thesis submitted by C. Chester Stock in partial fulfilment of the requirements for the degree of Doctor of Philosophy, the Johns Hopkins University. See preceding paper (3).

In this paper are recorded observations which depict the modifications induced in the activity-pH curve of liver arginase under certain conditions in the presence of manganous, cobaltous, or nickelous salts. Circumstantial evidence suggesting that the  $\alpha$ -amino group of *D*-arginine (*S*) may play a rôle in "anchoring" the postulated metallo-enzyme (*E*) in the sense of Equation 1



is strengthened by more direct evidence bearing upon the indispensability of this substituent group for the enzyme's *efficient* functioning. It is shown that the hydrolysis of neither  $\delta$ -guanidinovaletic acid nor argininic ( $\delta$ -guanidino- $\alpha$ -hydroxyvaletic) acid is effectively catalyzed by liver or jack bean arginase. The results reinforce the observations of other investigators in emphasizing the rather striking specificity in the enzymatic hydrolysis of *D*-arginine. This has prompted a comparison with the kinetics of hydrolysis of various guanidino compounds in the presence of an adequate concentration of hydroxyl ion to replace the enzymes as catalysts. Conditions are described for the controlled hydrolysis of *D*-arginine,  $\delta$ -guanidinovaletic acid, argininic acid, glycoeyamine, and guanidine. There have appeared important distinctions in the characteristics of the enzymatic and hydroxyl ion-catalyzed processes; in addition, the results have disclosed that *D*-arginine, as compared with other guanidines, possesses no unusual inherent instability toward hydrolysis.

Current studies in this laboratory are emphasizing also the nature of arginase as it occurs in tissue extracts.

#### EXPERIMENTAL

*Activity-pH Curves of Liver Arginase; Effect of Certain Heavy Metal Salts*—The preparation of crude arginase was carried out according to the directions of Hellerman and Perkins (2). It was found most convenient to reextract calf liver tissue several times and to combine the fluid extracts before going on with the several manipulations preceding the acetone precipitation. The use of glycerol is probably unnecessary. The use of a centrifuge before the precipitation step ordinarily may be omitted. Ample acetone is required in the drying process. From the dried acetone precipitate, *arginase solution* was prepared as previously directed.

The activity-pH curves were evaluated on the same basis as

that described by us previously (3). It was considered unnecessary at this time to try to employ a more ideal procedure. There is considerable variation in the rate of destruction of liver arginase with change in the hydrogen ion concentration in certain regions (4). With a reasonably "stable" enzyme preparation and the use of relatively short periods of hydrolysis, there were obtained data with which could be delineated the major differences in the activity curves.

The buffers used, the pH intervals at which observations were taken, the ionic strength in *d*-arginine-arginase mixtures, and the amounts of activating heavy metal salts added were all as specified in the preceding paper (3) in which we presented activity-pH curves of jack bean arginase.

The arginase solutions used varied somewhat in enzyme content. It was impracticable to make from one preparation simultaneous determinations of all the necessary data. However, in repeated determinations the zones of pH for optimal activity in the case of each enzyme preparation remained the same. In addition, the relative degree of activation referable to each of the several added metal ions was substantially as given in Fig. 1, which presents mean curves based on all the data.

*Procedure*—Water redistilled in glass was used. The reaction vessels were 25 ml. volumetric flasks of "resistance" glass. To a solution containing 10 ml. of buffer, 46 mg. of *d*-arginine hydrochloride, and appropriate heavy metal salt were added 5 ml. of arginase solution and water to make a total volume of 20 ml. After the further addition of 2 drops of caprylic alcohol, the mixture was kept at 37° for 3½ hours and then heated at 100° for 10 minutes (to destroy arginase). Adjustment to pH 7 was made and there were added 16 mg. of sodium cyanide, 0.1 gm. of crude urease, and 1 M phosphate buffer of pH 7.5 to the 25 ml. mark. A little caprylic alcohol was added and the mixture was permitted to stand at 30° for about 16 hours, after which it was filtered and the ammonia in 10 ml. of the filtrate estimated in the usual manner (2) in terms of ml. of 0.0200 N HCl neutralized.

Elaborate controls were carried out upon reagents and heat-denatured enzyme; the controls included determinations in which substrate or enzyme was omitted and estimations of pure urea under the conditions of the various runs. There were obtained blanks representing ammonia from the enzyme and reagents

equivalent to as much as 0.35 ml. of 0.0200 N HCl. Suitable corrections were applied throughout.

*Note on Estimation of Urea*—As pointed out by Stock, Perkins, and Hellerman, the activity-pH curves for the jack bean arginolytic enzyme suggest that the "arginase activity" of jack bean preparations, even in the presence of the activating ions,  $\text{Co}^{++}$ ,  $\text{Mn}^{++}$ , or  $\text{Ni}^{++}$ , may be abolished without gross impairment of the ureolytic activity if the hydrogen ion concentration of a reaction

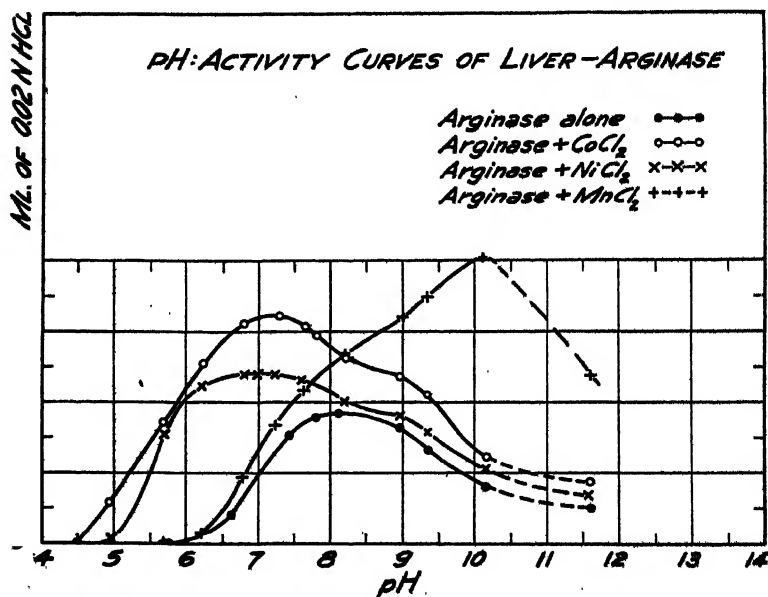


FIG. 1

mixture is adjusted to a value below pH 6.5. Dr. M. M. Richards finds<sup>1</sup> that liver arginase activity may be reliably evaluated or urea in the presence of *D*-arginine and "activating" salts accurately determined under conditions similar to those of the "Procedure" if the reaction mixture (after being heated and cooled) is treated with 16 mg. of sodium cyanide and then brought to pH 6 by the addition of hydrochloric acid before the addition of urease and buffer solution of pH 6. The use of cyanide is still found advantageous (2); hydrocyanic acid supports the action of urease (1). After

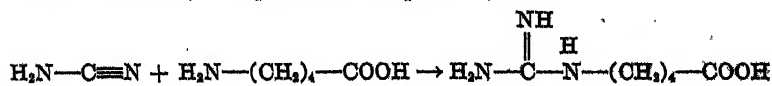
<sup>1</sup> Unpublished experiments.

2 hours at 30°, the reaction mixture is analyzed for ammonia by 2 hours aeration in the usual fashion. The titration mixture may be stirred with a stream of clean, CO<sub>2</sub>-free air, chlor-phenol red being a convenient indicator.

*Specificity of Arginase in Relation to Role of α-Amino Group in d-Arginine*—The work of a number of investigators has suggested that alterations in the *d*-arginine molecule (*e.g.*, esterification) prevent or markedly hinder the action of arginase. Inactivity has been observed with certain lower analogues and with some α-amino-substituted derivatives. In addition, the specificity concerned with the enantiomorphs of arginine is at least one of degree (5). Information concerning the essentialness of the α-amino group is conflicting or indecisive. It has been reported that one guanidino group of arginylarginine is hydrolyzed (6), that α, N-benzoylarginine is hydrolyzed at pH 6.5 to 7.5 (but not above or below this pH range) (7), and that α, N-methylarginine is not attacked (8). Observations of argininic acid hydrolysis in the presence of arginase were reported by Calvery and Block (9) and by Felix and Muller (10), although Felix, Muller, and Dirr (7) had failed previously to observe such hydrolysis.

The present investigation includes a study of the behavior of our preparations of liver arginase and of jack bean arginase, in the absence and in the presence of certain activating metal salts, with certain analogues of arginine (*δ*-guanidinovaleric acid and *δ*-guanidino-α-hydroxyvaleric (argininic) acid) *as compared with* the behavior of these enzymes with *d*-arginine under the same conditions.

*Preparation of δ-Guanidinovaleric Acid*—The method of preparation is that of Ackermann, Engeland, and Kutscher (11). They reported that an abundant crust of the compound is obtained after an ammoniacal solution containing specified amounts of cyanamide and *δ*-aminovaleric acid is allowed to stand for 5 weeks. The product was analyzed (N). According to conventional formulas, the process is depicted thus:



We proceeded as follows: *δ*-Aminovaleric acid hydrochloride, 5.06 gm., and cyanamide, 1.41 gm., were dissolved in the minimum amount of water in a 50 ml. beaker. The solution was made

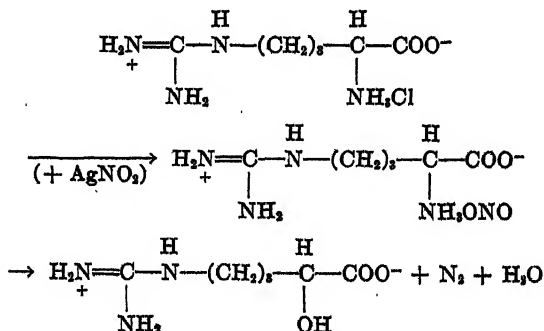


*slightly* alkaline with ammonia. After 3 weeks, hard clusters of crystals of  $\delta$ -guanidinovaleric acid had formed; these were collected, washed with 95 per cent ethanol, and crystallized from hot water. The solution gave a positive Sakaguchi test (12) and a negative chloride test. The product decomposed at  $273^\circ$ ; it was ground with absolute ethanol, freed of alcohol by filtration, and recrystallized from hot water. Two preparations yielded 4.62 gm. The capillary "melting point" was observed in a bath preheated to  $270^\circ$ ; decomposition point,  $283.5$ – $284.5^\circ$  (corrected). The previously reported melting point was  $265$ – $266^\circ$ .

	C	H	N	Ash
Calculated for $C_6H_{12}N_4O_2$ .....	45.25	8.2	26.4	
Found (Pregl microcombustions)*..	45.26	8.1	26.5	0.12

Sodium hydroxide may be used instead of ammonia to bring the reaction mixtures to slight alkalinity. From preparations in which were used 3.0 gm. of  $\delta$ -aminovaleric acid hydrochloride and an equivalent amount of cyanamide with sodium hydroxide there were obtained in 2 days yields of 1.0 to 1.5 gm. of  $\delta$ -guanidinovaleric acid. The products contained little ash.

*Preparation of Argininic Acid*—The method of Felix and Muller (13) was used. Some deviation was found necessary, owing to



the sluggishness of the reactions which involved the splitting of nitrogen (as  $\text{N}_2$ ) from arginine nitrite, and this has since been noted also by Felix and Muller (10). The method of preparation may be illustrated by the accompanying scheme.

A solution of 6.96 gm. of *d*-arginine hydrochloride and 5.57

\* For the microcombustions we are indebted to Dr. R. T. Milner and Mrs. H. S. Sherman of Washington, D. C.

gm. of silver nitrite in 350 ml. of water was stirred mechanically for an hour. The precipitated silver chloride was removed by filtration. The filtrate, which was found by test to be chloride-free, was saturated with hydrogen sulfide to remove remaining silver ions. After the removal of silver sulfide, the solution was concentrated under reduced pressure to about 50 ml. Ethanol was added until the mixture was slightly turbid; from the cooled mixture there were obtained 2.16 gm. of crystalline arginine nitrite. When ether was added to the mother liquor, there was obtained a second crop of 3.64 gm. Samples from both crops decomposed at 172–174°. The previously reported decomposition point was 160°. The theoretical yield was 7.3 gm. as compared with 5.8 gm. obtained. The product gave a positive test for nitrite with acidified potassium iodide. As a nitrite it also was found to reduce approximately the calculated amount of potassium permanganate.

Of arginine nitrite, 4.3 gm. were utilized for the preparation of argininic acid. The nitrite was found unusually resistant to decomposition when heated in an aqueous solution. It was found necessary to heat it in a concentrated aqueous solution with the use of a glycerol bath held at 120–130° for 5 to 8 hours; addition during this period of 50 ml. of 0.1 M HCl was also necessary to insure completion of the action. After the heating, the solution was evaporated to dryness under reduced pressure. The residual solid was taken up in the minimum amount of warm water, and the solution was treated with absolute ethanol and chilled. The yield of crystallized product was 1.17 gm. and of a less pure residue in addition, 0.75 gm. A solution of a sample of this argininic acid had a pH value of about 7.8. Tests for chloride and nitrite ion were negative.

This method for the preparation of  $\delta$ -guanidino- $\alpha$ -hydroxyvaleric acid is not regarded by us as entirely satisfactory or as fully conclusive of the structure of the product. A method of synthesis would be preferable in which is avoided the decomposition (14) of an amine nitrite grouping in a molecule having a guanidino substituent. However, estimations by the Van Slyke procedure indicated the presence in the product of 0.42 per cent or less of  $\alpha$ -amino nitrogen; the total nitrogen found (micro-Dumas) was 23.6 per cent as compared with 23.9 per cent cal-

culated. The product had in general the properties described by Felix and Muller. It was found also that the alkali-catalyzed hydrolysis of the compound yielded urea and ammonia and was comparable in rate with that of a number of other guanidino compounds. This is detailed in a later section.

For present purposes the configuration of the  $\alpha$ -carbon atom

TABLE I

*Liver Arginase with  $\delta$ -Guanidinovaleric Acid and with Argininic Acid*

Substrates are as follows: *D*-arginine hydrochloride,  $\delta$ -guanidinovaleric acid, and argininic acid, in amounts designated; the enzyme is liver arginase solution (described earlier in the paper and in (2)), 2 ml.; buffer in Experiments 1 to 3, 5 ml. of phosphate (1 M, pH 7.45) and in Experiments 4 to 6, 5 ml. of glycine (constituents as in Sørensen's, 1 M, pH 9.5); redistilled (in glass) water to a total volume of 20 ml.; caprylic alcohol, 2 drops; digestion, 20 hours at 37°; urea in two-fifths of the reaction mixture estimated as usual (see an earlier section). The results are expressed as ml. of 0.02 N HCl equivalent to ammonia from the urea formed and hydrolyzed.

Experiment No.	pH	Substrate*		0.02 N HCl neutralized	Substrate hydrolyzed
			mg.	ml.	per cent
1	7.45	<i>D</i> -Arginine hydrochloride	42	5.30	66
2	7.45	$\delta$ -Guanidinovaleric acid	32	0.00	0
3	7.45	Argininic acid†	35	0.44	0
4	9.5	<i>D</i> -Arginine hydrochloride	42	7.32	92
5	9.5	$\delta$ -Guanidinovaleric acid	32	0.00	0
6	9.5	Argininic acid†	35	0.44	0

\* Appropriate controls were made throughout. When substrate was absent, or enzyme was heated at 100° before addition, the ml. of HCl were 0.17 to 0.20. The values given have been corrected with these blanks. Total hydrolysis of two-fifths of the arginine hydrochloride used would produce urea ammonia equivalent to 8.00 ml. of 0.02 N HCl; of the  $\delta$ -guanidinovaleric acid, 7.83 ml.; of the argininic acid, 8.00 ml.

† Determination of  $\alpha$ -amino N (Van Slyke) indicated that the argininic acid used contained sufficient *D*-arginine to yield ammonia equivalent to 0.42 ml. of 0.02 N HCl.

with respect to asymmetry was neglected. (The starting material for argininic acid was, as stated, *D*-arginine. In accordance with the more usual practice, the designation *D*-arginine has been used in our papers to refer to the "natural" enantiomorph.)

*Results of Hydrolysis Experiments*—The details are recorded in Tables I to IV.

TABLE II

*Liver Arginase and Cobaltous Chloride with  $\delta$ -Guanidinovaleric Acid and with Argininic Acid*

Substrates, those of Table I and *d*-arginine nitrite; liver arginase solution, 2 ml.; phosphate buffer (1 M, pH 7.45), 5 ml.; cobaltous chloride added where indicated; water to 20 ml.; caprylic alcohol, 2 drops; digestion, 3 hours at 37°; urea estimated and results expressed as in Table I.

Experiment No.	CoCl <sub>2</sub> ·6H <sub>2</sub> O	Substrate*	0.02 N HCl neutralized	Substrate hydrolyzed
	mg.	mg.	ml.	per cent
1		<i>d</i> -Arginine hydrochloride 42	3.09	39
2	2	" " 42	6.41	80
3		" nitrite 44	3.10	39
4	2	" " 44	6.50	82
5		$\delta$ -Guanidinovaleric acid 32	0.00	0
6	2	" " 32	0.00	0
7		Argininic acid* 35	0.30	0
8	2	" " 35	0.33	0

\* See foot-notes of Table I.

TABLE III

*Jack Bean Arginase and Cobaltous Chloride with  $\delta$ -Guanidinovaleric Acid and with Argininic Acid*

Substrates, as indicated (see Table I); enzyme, crude jack bean urease, 0.1 gm. (or crude crystalline urease of comparable argininolytic activity); buffer, in Experiments 1 to 4, 5 ml. of phosphate (1 M, pH 7.45) and in Experiments 5 to 8, glycine (1 M, pH 9.50); cobaltous chloride added where indicated; water to 20 ml.; caprylic alcohol, 2 drops; digestion, 20 hours at 30°. After digestion, the mixture was diluted to 25 ml. and ammonia estimated in a 10 ml. portion as usual (see text). The results are expressed as in Table I.

Experiment No.	pH	CoCl <sub>2</sub> ·6H <sub>2</sub> O	Substrate*	0.02 N HCl neutralized	Substrate hydrolyzed
		mg.	mg.	ml.	per cent
1	7.45		<i>d</i> -Arginine carbonate 45	0.45	5
2	7.45	2	" " 45	7.76	89
3	7.45	2	$\delta$ -Guanidinovaleric acid 32	0.00	0
4	7.45	2	Argininic acid* 35	0.06	0
5	9.5		<i>d</i> -Arginine carbonate 45	2.16	25
6	9.5	2	" " 45	4.08	46
7	9.5	2	$\delta$ -Guanidinovaleric acid 32	0.07	0
8	9.5	2	Argininic acid* 35	0.44	0

\* See foot-notes of Table I.

From the results given in Table I, it is seen that under conditions similar to those more usually employed in this series of investigations, *D*-arginine in the presence of liver arginase was extensively hydrolyzed in 20 hours in reaction mixtures of pH 7.45 or 9.5, while the hydrolysis of the closely related compounds,  $\delta$ -guanidinovaleric and argininic acids was quite negligible. Similarly, the data of Table II emphasize that under conditions in which the arginase preparation used was normally activated in the presence of cobaltous ion so that *D*-arginine, introduced as hydrochloride or nitrite, was 80 per cent hydrolyzed in 3 hours at 37°, the two related guanidino compounds, in contrast, were not observedly changed. With jack bean argininolytic enzyme and cobalt chloride under conditions suitable for the extensive hydrolysis of *D*-arginine during 20 hours, there was observed no hydrolysis of the other two substrates (Table III).

Since, however, from a chemical standpoint enzyme specificity probably is not an absolute characteristic but rather one of degree, it was considered desirable to test the specificity of liver arginase with these closely related analogues of arginine under extreme conditions; *i.e.*, with 100 times the amount of enzyme usually employed together with cobaltous and manganous salts, in favorable pH regions, and during a long digestion period. These procedures appeared, in a measure to "force" the enzymatic hydrolysis of  $\delta$ -guanidinovaleric and argininic acids. Table IV brings out that in 65 hours the former compound was hydrolyzed to the extent of 8 to 14 per cent and the latter, 25 to 38 per cent. Contrarily, there has been effected, in less than one-twentieth of this time, with a greatly diminished amount of enzyme, the hydrolysis of *D*-arginine to the extent of 80 per cent of its initial concentration. The significance of these results will be discussed later.

#### *Hydrolysis of Guanidino Compounds in Alkaline Solution*

*Non-Catalysis by Certain Heavy Metal Salts and Hydroxides*—The ions,  $\text{Fe}^{++}$ ,  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ , and  $\text{Mn}^{++}$ , which have been considered to participate through coordination in the enzymatic hydrolysis of *D*-arginine do not of themselves function as catalysts in this process. These ions alone appear to be entirely devoid of catalytic activity under the conditions of this series of investigations upon arginase. This is based upon the results of numerous

control experiments in which the enzyme was omitted in arginase-heavy metal salt studies. Equally without any striking effect are these ions or the hydroxides derived from them in the alkali-catalyzed hydrolysis of *d*-arginine. When, for example, there

TABLE IV

*Specificity of Calf Liver Arginase: More Extreme Test with  $\delta$ -Guanidinovaleric and Argininic Acids*

Enzyme, liver arginase solution, 2 ml. (extract of 100-fold the dried arginase preparation usually used; *i.e.*, 11 gm. of powder extracted with 50 ml. of water); buffer, 5 ml., of such strength that in the final reaction mixture (10 ml.), ionic strength,  $\mu$ , equaled unity: in Experiments 1 and 3, phosphate of pH 7.4 and in Experiments 2 and 4 glycine of pH 9.2; activating metal salt,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (Co) or  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (Mn), 2 mg., as indicated; water to 10 ml.; caprylic alcohol, 0.1 ml.; toluene, 0.2 ml.; temperature 37°; time 65 hours. Digestion was carried out in aeration tubes and the estimation with urease was made for the urea in the entire mixture; the results are expressed accordingly. Substrates are as indicated (*cf.* Table I). In parallel experiments under similar conditions there was hydrolyzed 93 per cent of an amount of *d*-arginine equivalent to the argininic acid or  $\delta$ -guanidinovaleric acid used in the tabulated determinations.

Experiment No.	Metal salt	Substrate*	0.02 N HCl neutralized†	Substrate hydrolyzed
			mg. ml.	per cent
1	Co	$\delta$ -Guanidinovaleric acid	11.56	0.58
2	Mn	" "	11.24	0.99
3	Co	Argininic acid‡	13.1	1.90
4	Mn	" "	13.1	2.89

\* Complete hydrolysis of the argininic acid would have produced urea ammonia equivalent to 7.60 ml. of 0.02 N HCl; for the  $\delta$ -guanidinovaleric acid, 7.28 and 7.08 ml. of 0.02 N HCl for Experiments 1 and 2 respectively.

† The results have been corrected on the basis of appropriate controls. Under the conditions of these experiments the controls are larger than usual. The magnitude of the blanks is lowered when the procedure suggested in "Note on estimation of urea" is followed.

‡ See "Preparation of argininic acid" and Table I.

was added to a 0.005 M solution of *d*-arginine hydrochloride a nickelous or cobaltous salt followed by adjustment with sodium hydroxide to pH 8.5 or 9, after which the solution was permitted to stand at 37° for 3 weeks, a suitable test disclosed no hydrolysis of the guanidino acid. Moreover, manganous salts failed to effect

any hydrolysis of 0.008 M *d*-arginine dissolved in a 0.1 M glycine buffer of pH 8.5 when the mixture was held at 37° for 4 weeks.

The rate of hydrolysis of *d*-arginine or guanidine in an alkaline solution becomes appreciable above pH 10.5 even when the reaction is observed at 35–40°. Under such conditions there is no observable change in rate in the presence of nickelous hydroxide.

*Hydroxyl Ion-Catalyzed Hydrolysis of d-Arginine and Other Guanidino Compounds*—Observations were made with *d*-arginine hydrochloride,  $\delta$ -guanidinovaleic acid, argininic acid, glycoylamine (guanidinoacetic acid), and guanidine. With each substance was prepared a solution 0.008 M with respect to the guanidino compound and 0.1 M with respect to potassium hydroxide. The solutions were kept in tightly stoppered, 1 liter Erlenmeyer flasks at  $37.8^\circ \pm 0.2^\circ$ . Because of the low rate of the hydrolyses, these conditions were considered adequate for comparative purposes. As controls were used a solution of KOH (0.1 M) and solutions of the following, each of which was also 0.1 M with respect to KOH: 0.008 M urea, 0.004 M ammonium sulfate (*i.e.*, the salt was added to the solution of KOH), 0.008 M *d*-ornithine, 0.008 M glycine. To each of the solutions were added caprylic alcohol, 0.3 ml., and toluene, 0.3 ml. Hydrogen electrode determinations of the pH initially and after the final hydrolysis measurements showed all the values to be  $\text{pH } 12.58 \pm 0.02$ .

At selected intervals two 10 ml. samples were taken from each flask. One sample was used at once for the determination of ammonia by aeration. The second sample was subjected to treatment with urease before aeration, the result giving total ammonia (*i.e.*, free  $\text{NH}_3$  + urea  $\text{NH}_3$ ). For this purpose, the sample was brought to pH 7.5 with HCl; 6 ml. of phosphate buffer (1 M, pH 7.49) and 0.05 to 0.10 gm. of crude urease were added, after which the mixture was held 2 hours at 30°. This was followed by the usual aeration procedure. The presence of urea in the hydrolysis mixtures was shown also by tests with xanthidrol (crystallization of dioxanthidrol urea).

*Results*—The analytical data are summarized in Table V. It is seen that the determinations of urea were sufficiently accurate and that there occurred little or no loss of ammonia from the reaction flasks. There was formed no ammonia in the urea, glycine, or *d*-ornithine controls. *Ammonia did appear in the reaction mix-*

tures. Its absence in the urea, glycine, and *d*-ornithine controls indicated that the ammonia formed during the hydrolysis of the guanidino compounds was derived from some source other than a secondary decomposition of urea or a residual amine. Accordingly, it appeared likely that in addition to the main reaction leading to urea and an amine, there was occurring also a simultaneous hydrolysis of the substituted guanidino compounds to yield ammonia and substituted ureas (*e.g.*, citrulline from *d*-arginine). A similar hydrolysis of a substituted guanidine by two paths was studied by Neubauer (15) who found (*cf.* (16)) that the decomposition of creatine by hot barium hydroxide gave sarcosine and urea together with methyl hydantoin and ammonia. Likewise, Schotté (17) found that creatinol,  $\text{H}_2\text{N}-\text{C}(=\text{NH})-\text{N}(\text{CH}_3)-(\text{CH}_2)_2\text{OH}$ , with *n* alkali at room temperature yielded ammonia, urea, *N*-methyl-*N*, $\beta$ -hydroxyethylurea, and *N*-methylaminoethanol. The data obtained in the present study have been analyzed on the basis of two concurrent reactions in each hydrolysis experiment. These statements are applicable to the substituted guanidino compounds and not to the case of guanidine, itself, of which the products of controlled hydrolysis are solely ammonia and urea.

Some data on the alkaline hydrolysis of guanidine were presented by Bell (18); calculations based on these data indicate that the process studied by him was essentially pseudomonomolecular. The enzymatic hydrolysis of *d*-arginine under certain conditions has also been shown (2, 3) to proceed pseudomonomolecularly. The data of the alkaline hydrolyses of this investigation (treated as irreversible reactions) were tested by substitution in a first order equation. The rate of conversion of a substrate compound is formulated as  $-ds/dt = ks$ , whence  $k = (2.303/t) \times (\log a - \log s)$ , where  $k$  = reaction constant,  $a$  = initial substrate concentration, and  $s$  = substrate concentration at time,  $t$ .

The reaction constant  $k$  would equal the sum of the constants for the two simultaneous hydrolyses, the rate of each of which is dependent upon the substrate concentration. Thus,  $k = k_1 + k_2$ , where  $k_1$  and  $k_2$  are the reaction constants for the processes that lead to ammonia and to urea, respectively. The ratio of these reaction constants should be directly proportional to the ratio of the molar equivalents of the respective products of hydrolysis formed in the two processes at any time,  $t$ .



TABLE V

*Hydroxyl Ion-Catalyzed Hydrolyses: Determinations of Ammonia and "Total Ammonia"*

Temperature, 37.8°; pH 12.58; all values for  $k$  are in inverse hours;  $k_1/k_2$  shows average values of ratios of the relative rates for the two paths of hydrolysis; the last column, values of  $k$  from the slope of the curve in the graph of  $\log s$  with respect to time.

The data for A. and T.A. are expressed as ml. of 0.02 N HCl neutralized by the  $\text{NH}_3$  formed. A. is equivalent to ammonia directly formed; T.A. to total ammonia (free  $\text{NH}_3$  + urea  $\text{NH}_4$ ) (see text).

Substrate*	Initial amount in 500 ml.	gms.	Time, hrs.										$\frac{k_1}{k_2}$		$k$
			0	28	48.5	91.75	141	215	354.5	522.25	691				
<i>D</i> -Arginine hydrochloride	0.8432	A.	0.03	0.07	0.07	0.15	0.25	0.28	0.37	0.51	0.50				
		T.A.	0.05	0.48	0.86	1.58	2.22	3.21		5.36	6.05	0.21		0.00259	
		$k \times 10^5$		250	258	268	263	268		254	248				
$\beta$ -Guanidinopivalic acid	0.6365	A.	0.06	0.04	0.04	0.15	0.19	0.24	0.32	0.40	0.38				
		T.A.	0.01	0.53	0.88	1.70	2.39	3.41	4.68	5.65	6.58	0.17		0.00286	
		$k \times 10^5$		268	258	296	284	291	288	286	320				
Glycoeyamine	0.4683	A.	0.05	0.10	0.08	0.22	0.44	0.50	0.67	0.92	0.97				
		T.A.	0.03	0.50	0.86	1.58	2.28	3.04	4.31	5.19		0.40		0.00275	
		$k \times 10^5$		279	264	278	295	272	275	277					
Guanidine carbonate	0.3642	A.	0.05	0.53	0.88	1.01	2.07	2.67	3.34	3.49	3.86				
		$k \times 10^5$		512	507	485	511	509	499	382	459			0.00510	
		T.A.	0.04	1.45	2.50	4.44	6.01	8.04	10.00	10.92					
Arginine acid	0.7032	A.		455	477	499	487	508	493	446				0.00499	
		$k \times 10^5$		0.04	0.07	0.04	0.08	0.20		0.32	0.45				
		T.A.	0.04	0.59	0.92	1.76	2.42		4.45		6.32	0.16		0.00279	
		$k \times 10^5$		311	278	288	285	281		281					

Urea, control	0.2395	A.	0.06	0.04	0.03	0.05	0.05	0.09	0.07	0.17	0.16
		T.A.	7.75	7.74	7.85	7.96	7.78	7.93	7.86	7.78	7.92
Ammonium sulfate, control	0.2650	A.	3.97	3.98	3.99	3.92	3.99	3.96	3.88	3.92	3.87
		T.A.	3.81	3.93	3.88	3.92	3.82	3.96	3.90	3.88	3.89
KOH, 0.1 N, control		A.	0.03	0.00	0.00	-0.01	-0.02	-0.02	-0.04	0.05	0.03
		T.A.	-0.02	0.00	-0.01	0.01	0.02	0.02	-0.07	0.01	0.01
Per cent†.....					98.7	99.7	96.9	99.9	100.1	99.0	99.9

\* The purity of these materials was determined by analysis.

† Per cent of urea estimated in parallel controls (4.5 mg. of urea) which were carried out with each series of determinations.

$$\frac{k_1}{k_2} = \frac{\text{moles NH}_3 \text{ formed}}{\text{moles urea formed}}$$

The analytical results (Table V) are given in terms of ml. of 0.0200 N HCl neutralized by ammonia. The values of  $a$  may be formulated conveniently in terms of ml. of 0.0200 N HCl equivalent to the amount of guanidino compound initially present in 10 ml. of a reaction mixture. For an 0.008 M solution, this would be 0.0008 mole  $\approx$  4.00 ml. of HCl. Of a substrate, the moles

### LOG S

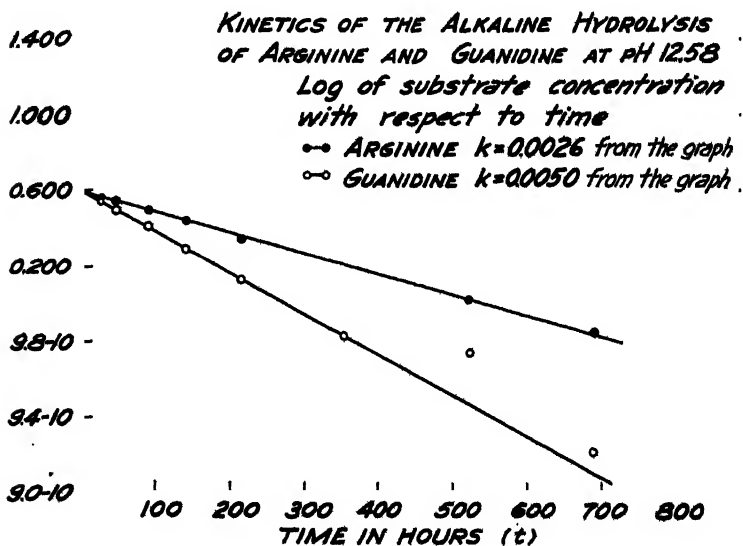
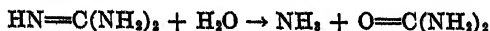


FIG. 2

hydrolyzed at time  $t$  are equivalent to the sum of moles of  $\text{NH}_3$  and moles of urea formed; this may be given in terms of the sum of A. (Table V), the ml. of 0.02 N HCl neutralized by  $\text{NH}_3$ , and U., which is one-half the ml. of HCl neutralized by the urea  $\text{NH}_3$  (obtained by urease hydrolysis). Thus,  $U. = \frac{1}{2} (T.A. - A.)$  and  $s = a - (A. + U.)$ . T. A.  $\approx$  "total ammonia" in 10 ml.

However, in the case of guanidine carbonate,  $a$  is taken alternatively as 4.04 ml. or as 12.11 ml., depending upon the choice of A. or T.A.; respectively, as equivalent to moles of guanidine hy-

dolyzed at time  $t$ . This is permissible, since the hydrolysis of guanidine here may be considered to involve only the reaction



The data depicted in Table V and Fig. 2 disclose (1) pseudomonomolecularity in the hydrolysis of each of the guanidino compounds, observed during a period of almost a month, (2) reaction constants ( $k$ ) of the same order of magnitude in all cases (the magnitude of  $k$  for guanidine exceeding that for any of the substituted guanidines studied), (3) comparable ratios for the  *$\delta$ -guanidino-substituted acids* of  $k_1/k_2$ , the reaction constants respectively for the ammonia- and urea-producing paths.

#### DISCUSSION

Certain aspects of enhancement of liver arginase activity in the presence of activating metal salts are emphasized in the several pH-activity curves (Fig. 1). In the presence of cobaltous or nickelous ion (but not of the potently activating manganous ion) there results a marked shift in the optimal pH zone for our preparations of liver arginase, connoting a rather wide extension of the range of effective action into the more acid region. We consider it not improbable that this phenomenon reflects, *in part*, the relative ability of the postulated enzyme-metallous ions concerned to form coordinate bonds, for example, with appropriate groups of the substrate molecules.<sup>2</sup> This may approximate the corresponding behavior of the effective heavy metal ions, themselves, which under certain conditions (Fig. 3) have been found to follow, qualitatively, the order  $\text{Ni}^{++} > \text{Co}^{++} > \text{Mn}^{++}$ . Elsewhere, the matter has been elaborated in some detail (3). It is to be noted that relatively more emphasis is accorded the data summarized by the pH-activity curves *in the less alkaline region* than in the sections of higher pH value; this applies in

<sup>2</sup> The discussion neglects the question of the relative stability of postulated donor-enzyme-metal complexes in different pH regions; it neglects, further, the effect of their varying stability upon the activity-pH relations. It may be stated that unpublished dialysis experiments in this laboratory strongly indicate a significantly greater stability (at pH 7) for cobaltous- or nickelous-arginase than for manganous-arginase (Richards, unpublished experiments).

particular to the detail of the *manganous*-enzyme curve in the alkaline region exceeding pH 9, since the controls in this region gave rather large blanks.

The results of this study make apparent one factor underlying the differences in the pH-activity curves of arginase (and certain other (1) enzymes) as developed by different observers. Thus, the character and quantity of heavy metal activator ions present in the crude enzyme preparations employed must determine to some extent the characteristics of the activity curve. In consequence, it is not surprising that an activity curve (19, 20) evaluated for the arginase preparations used by Hunter and his collaborators resembles somewhat the arginase-manganous curve presented here (Fig. 1). Of pertinence to the discussion are the experiences of various workers. Hunter and Morrell first (21) reported an optimum at pH 7.2, but later (19) amended their conclusions and placed the optimum at about pH 9.8. Hino (22) found the optimum for his preparation at pH 7.3 to 7.5, while Edlbacher and Bonem (23) reported pH 9.5 to 9.8; Edlbacher and Simons later indicated an optimum near pH 9.0 (24).

The identity of the metallic component of, or coenzyme (1) that cooperates *in vivo* with, natural liver arginase is at present a matter of conjecture. There may be involved one or more of the heavy metal constituents of liver cells such as  $\text{Fe}^{++}$  or  $\text{Mn}^{++}$  or a metallo-organic entity.<sup>3</sup> Presumably arginase is a protein. Edlbacher and Pinösch (27) now stress the significance of manganous ion for its activation (*cf.* (5)).

Although intensive labor of fractionation will be required to gain further insight into the nature of arginase, the present position has permitted the construction of a useful working theory concerning a phase of its mode of action (1-3). Only certain aspects need be stressed here. The most effective *in vitro* activators

<sup>3</sup> The fact that *d*-ornithine in contradistinction to urea slows the rate of *d*-arginine hydrolysis in the presence of arginase is not surprising (*cf.* (25) p. 929). Practically, the hydrolysis is irreversible. The activity of the arginase *itself* doubtless is diminished by *d*-ornithine under certain conditions. A similar effect would not be anticipated for urea; suitable titrations demonstrate (26) that *d*-ornithine readily enters into metallo complex formation.

seem to be reduced ions of certain metals of the first transition series in the periodic table. The characteristics of inhibitions and reactivations of liver arginase suggest that catalysis by arginase involves metal-coordinated complexes. Furthermore, the observations concerned with titrations of *d*-arginine in the presence of certain heavy metal salts and the shifts in pH-activity curves together may be taken as an indication that the  $\alpha$ -amino group of *d*-arginine may be concerned in the elaboration of dissociable catalyst-substrate complexes through metallo coordination. Elsewhere this has been depicted on a crude, tentative basis (1).

Our representation admittedly has been incomplete. For example, one requirement for effective arginase action appears to be a carboxyl anion in the substrate; esters of *d*-arginine are reported unattacked (7, 9, 23). It seems most probable that the carboxyl anion in addition to the  $\alpha$ -amino group is involved in the orientation of substrate to metallo-enzyme, somewhat similarly to its involvement in simpler  $\alpha$ -amino acid metallo complexes. In addition, any complete picture must account more adequately for the importance of geometric configuration.<sup>4</sup>

We have proposed (1, 2) that the modification of resonance ((25) p. 1872) in the guanidinium ion of *d*-arginine might account most fundamentally for the unique action of the enzyme arginase. Earlier we diagrammed this as resulting from the formation of a

<sup>4</sup> Compare the reported (28) non-hydrolysis of *dl*- $\epsilon$ -guanidino- $\alpha$ -aminocaproic acid (homoarginine). Arginase is not an effective catalyst in the hydrolysis of guanidine or certain simple derivatives (29), including  $\gamma$ -guanidinobutyric acid ((7, 23, 30), cf. (31) (25) p. 929)) regarding which conflicting statements have appeared. On the contrary, canavanine,  $^+H_2N=C-N(H)-O-(CH_2)_2-C(H)-COO^-$ , which from a purely struc-



tural view-point is *d*-arginine in which a methylene grouping is replaced by hydroxylamino oxygen, is hydrolyzed (32) in the presence of a liver enzyme to urea and canaline. It is conceivable that the argininolytic enzymes are identical with the canavanases (cf. (3) foot-note 5). The properties of canavanases will be studied in this laboratory. Difficulties which have been experienced in the evaluation of results published by different authors in the field of enzyme specificity are often due to the omission of even a semiquantitative treatment of the relative efficiency of the enzyme with respect to the substrates considered.

coordinate link between metal ion and substituted guanidinium nitrogen. In the absence of supporting direct evidence we prefer now to submit only that the character of the resonance could be altered through some appropriate addition reaction involving the guanidino group and a grouping of the enzyme molecule. On this basis, the effect would involve a "weakening" of the bond shared by the ion's central carbon atom and the substituted nitrogen atom (*i.e.*, N attached to the side chain) and, possibly, a concomitant strengthening of the bonds with the other 2 nitrogen atoms, through intensified resonance involving them. An extended discussion of this and alternative possibilities regarding the mechanism of arginase action would be premature.

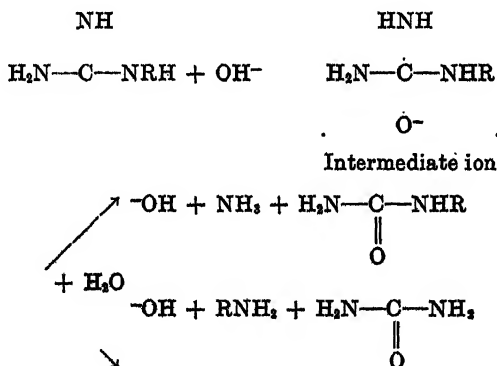
Assumptions concerning the rôle of the  $\alpha$ -amino group of *D*-arginine in the *orientation of enzyme to substrate* are supported by tests of the specificity of arginase. The results recorded in the experimental part strengthen the opinion that the  $\alpha$ -amino group is required for the *efficient* functioning of the enzyme. The catalytic efficiency of arginase with *D*-arginine, on the one hand, and  $\delta$ -guanidinovaleric or argininic acid, on the other, is manifestly of a very different order. As between the latter two substrates the action of arginase with argininic acid seems slightly more noticeable, owing possibly to an enhanced associating effect attributable to the  $\alpha$ -hydroxyl group.<sup>5</sup>

That arginase can be demonstrated, under extreme conditions, to display catalytic activity with these substances or with *L*-arginine (5) is not surprising in view of current concepts of enzymatic and general catalysis. For example, the postulated catalyst-substrate complex (*ES* in Equation 1) is considered to be the species chiefly undergoing hydrolytic change with regeneration of enzyme (*E*). If certain guanidino compounds structurally related to *D*-arginine may be assumed to enter into equilibria analogous to the process shown in Equation 1, the efficiency of the enzyme with respect to their hydrolysis may be related importantly to the magnitude of the association constants. Thus, with the use of a relatively large amount of enzyme and a sufficiently long

<sup>5</sup> It has been reported (7) that  $\alpha$ ,N-benzoylarginine is hydrolyzed under certain special conditions. Octopine is unhydrolyzed (33) in the presence of arginase or is noticeably attacked (34) after long reaction periods. The  $\alpha$ -uramido derivative of *D*-arginine is attacked (20), but much less efficiently than the parent amino acid.

reaction time there might result measurable hydrolysis. Then in a special sense "specificity" of the enzyme becomes a matter of degree. The advantage of a dissociable intermediate complex in which metallo-enzyme has been bound through coordination to the substrate's  $\alpha$ -amino group and carboxyl anion would reside not merely in the provision for favorable association of enzyme and substrate, but, further, in an accompanying strategic orientation of the enzyme's "working group" with respect to the guanidinium ion.

The comparison of an enzymatic hydrolysis with a somewhat comparable non-enzymatic process has proved instructive. The salts of guanidines are ordinarily highly resistant to hydrolysis, even at elevated temperatures. Contrarily, the parent bases are more or less readily hydrolyzed with hydroxyl ion as catalyst.



In this study, there was observed the controlled non-enzymatic hydrolysis of *d*-arginine together with the like hydrolysis of a number of other guanidines, none of which is readily hydrolyzed with arginase as catalyst. The results are given in the experimental section. In contrast to the striking specificity of the argininolytic enzymes, there has been revealed the similarity in the kinetics of alkaline hydrolysis of the entire series, including *d*-arginine. In contrast, also, to the experiences of a number of workers with the enzymatic process, which seemingly produces from *d*-arginine only urea and *d*-ornithine, there had to be considered two paths for the alkaline hydrolysis of substituted guanidines. This has been discussed earlier; it is illustrated by the accompanying dia-



gram which includes a schematic representation of the mechanism of the alkaline hydrolysis.

This raises a question whether, in addition to its other unique attributes, most conspicuously, the efficient catalysis of *d*-arginine hydrolysis in the regions of hydrogen ion activity where the substrate is otherwise notably "stable,"<sup>6</sup> arginase possesses the property of directing the course of the hydrolysis practically exclusively in one path. It may be stated that with specificity of this character our suggestions regarding a possible correlation of the enzyme activity with alteration of resonance in the guanidinium ion of *d*-arginine seem entirely consistent.

#### SUMMARY

1. Exploratory activity-pH curves for certain preparations of liver arginase have revealed in greater detail some of the aspects of the enzyme action in the presence of heavy metal activator ions. The results are considered to indicate an important source of divergence in pH-activity curves of certain enzymes as developed by different workers. It is proposed that the data *taken with other observations* suggest the participation of the  $\alpha$ -amino group of *d*-arginine in the orientation of enzyme to substrate through metallo complex formation.

2. There has been investigated the specificity of arginase, particularly in relation to the rôle of the  $\alpha$ -amino group of *d*-arginine. In this connection are described modifications in the preparation of  $\delta$ -guanidinovaleric and argininic acids. The question has been raised to what extent enzyme specificity may be a matter of degree rather than an absolute property.

3. A comparison has been made of the characteristics of the enzymatic hydrolysis of *d*-arginine and of the controlled alkaline (non-enzymatic) hydrolysis of *d*-arginine,  $\delta$ -guanidinovaleric acid, argininic acid, glycocyamine, and guanidine. The results have disclosed important distinctions in the differently catalyzed processes. The unique qualities in the action of arginase are discussed in relation to an idea which visualizes a correlation of the

<sup>6</sup> The stability to hydrolysis of the salts of guanidine and certain of its derivatives, in contrast to the parent guanidino bases, has been attributed to a high degree of resonance in the symmetrical guanidinium ions.

enzyme action with the alteration of resonance in the guanidinium ion of *D*-arginine.

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# THE SIMULTANEOUS DETERMINATION OF TOTAL BASE AND CHLORIDE ON THE SAME SAMPLE OF SERUM BY ELECTRODIALYSIS

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An electrodialytic method for the determination of total base in serum and other biological fluids has been recently described by Keys (5), whose procedure is based on the earlier work of Stoddard (7) and Adair and Keys (1). Mercury within a cylindrical cathode vessel is separated from the material to be analyzed by a cellophane membrane. As the electric current is transported, the diffusible cations form an amalgam, which is allowed to react with standard acid. Upon completion of the electrodialysis, the acid within the cathode vessel is titrated with standard alkali. The total base in the sample is estimated as equivalent to the displaced hydrogen. Determination of total base by this method is rapid and eliminates the ashing required in the Fiske (3) and Stadie-Ross (6) methods.

A modification of the Adair-Keys method is here presented by which the total base and chloride are simultaneously determined in the same sample of serum. An anode chamber, separated by a cellophane membrane from the middle chamber containing the serum, is added to the system. After electrodialysis, the total base in the cathode chamber is determined as in the Adair-Keys method, and the chloride, collected in the anode chamber free of protein, is determined by the Volhard method (10).

The successful quantitative determination of chloride by electrodialysis depends upon the use, in the anode chamber, of a reducing agent to prevent the oxidation of chloride ion into free chlorine which would escape. Control analyses showed that, in the absence of such a reducing agent, up to 50 per cent of the

chloride might be lost. The reducing agent completely prevents loss of  $\text{Cl}^-$  and 100 per cent recovery is possible. It was found that glucose in dilute acetic acid served admirably as the reducing agent to be used in the anode chamber.

A somewhat similar method of electrodialysis has been employed by di Benedetto (2), who was able, however, to effect only partial recovery of base.

### *Apparatus and Procedure*

The apparatus employed is illustrated in Fig. 1. It consists essentially of three vessels, a beaker (A), about  $1\frac{1}{2}$  inches in diameter, which serves as the anode vessel, and two concentric cylinders (B and C), respectively about  $\frac{7}{8}$  inch and  $\frac{3}{4}$  inch in diameter. The latter are arranged as in the illustration, the larger cylinder supported in the beaker by wire attached near the upper end, while the smaller cylinder can be suspended in the larger by the same means. The anode solution contains dilute acetic acid, as electrolyte, and glucose, added to reduce any free chlorine at the anode. The larger cylinder contains the material to be analyzed, while the smaller one is the cathode vessel. The lower end of each cylinder is enclosed by a cellophane membrane. A square piece of du Pont's No. 300 cellophane is moistened in water for a few minutes, then dried, and stretched tightly over the end of the cylinder. After the membrane is held firmly in place for a short time, the upper edges are trimmed with a sharp knife or scalpel, and are fixed to the cylinder by means of collodion.

An electrodialysis is carried out in the following manner. Approximately 5 cc. of electrolyte solution are introduced into the anode vessel. We have used 0.05 N acetic acid plus 3 per cent glucose for this purpose. Approximately 0.5 cc. of purified mercury is introduced into the cathode vessel and covered with 2 cc. of 0.1 N hydrochloric acid. A platinum cathode makes contact with the mercury. 0.5 cc. of serum or unknown solution is pipetted into the larger cylinder, and is diluted with about 5 cc. of water. The concentric cylinders are then suspended in the anode vessel, a platinum anode is inserted into the anode solution, and electrolysis is permitted to proceed. 110 volts direct current

are used, passing through an external resistance of about 1000 ohms. The electro dialysis is carried out for about 1 hour, at the end of which time the anode and cathode vessels are removed and their contents titrated. The anode solution, as well as the cathode solution, is protein-free, and chloride can be determined without the necessity of ashing.

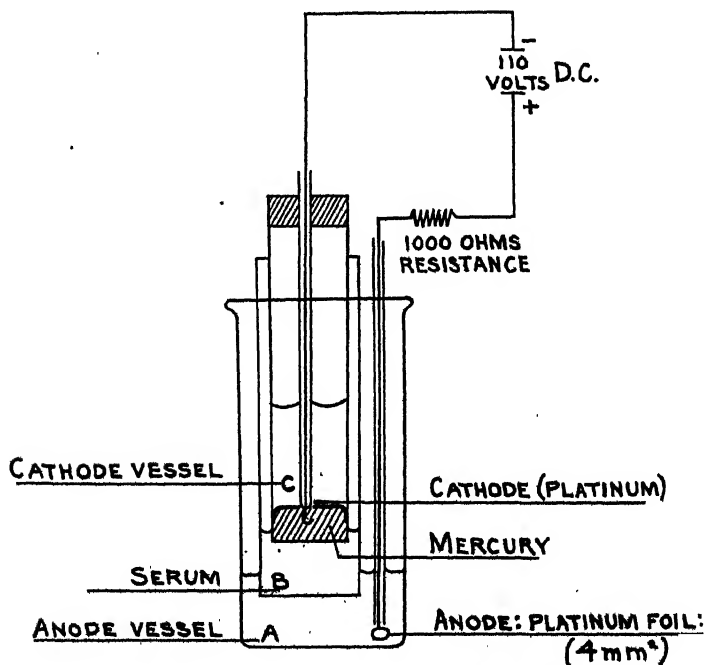


FIG. 1. Electro dialysis apparatus

### *Analysis*

Total base is determined by titration of the contents of the cathode vessel with standard alkali, a burette graduated to 0.05 cc. being employed. A convenient concentration of standard alkali is 0.025 N. The total base expressed in milliequivalents per liter of serum is given by the expression

$$\text{Total base} = \frac{1000}{0.5} (0.2 - 0.025 \times \text{cc. NaOH})$$

Chloride is determined by titrating the contents of the anode vessel according to the Volhard method (10). It is precipitated as silver chloride by the addition of 1 cc. of 0.15 N silver nitrate. After removal of the precipitate by filtration the excess silver is titrated with 0.02 N potassium thiocyanate, with ferric alum as

TABLE I  
*Comparison of Total Base and Chloride As Determined by  
Independent Methods*

The figures represent the mean values and standard deviations of a series of determinations. The results are expressed in milliequivalents per liter.

Horse serum	No. of determinations	Base	Chloride	Method
A	16	154.5 $\pm$ 0.7	104.3 $\pm$ 0.3	Electrodi- alysis
	6	152.7 $\pm$ 0.7	104.5 $\pm$ 0.2	Ashing*
A + 0.5 cc. 0.05 N NaCl	6	205.2 $\pm$ 0.7	154.2 $\pm$ 0.4	Electrodi- alysis
		204.5 $\pm$ 0.7	154.3 $\pm$ 0.3	Calculated
B	12	151.6 $\pm$ 0.8	103.0 $\pm$ 0.5	Electrodi- alysis
	6	149.5 $\pm$ 0.7	103.0 $\pm$ 0.4	Ashing*
A	16	162.0 $\pm$ 0.6	107.0 $\pm$ 0.4	Electrodi- alysis
	16 Base 6 Chloride	161.0 $\pm$ 0.7	106.8 $\pm$ 0.4	Ashing†

\* Base was determined by the Stadie-Ross (6) procedure on 0.5 cc. samples; chlorides by the Wilson-Ball (11) method on 0.5 cc. samples.

† Base was determined as in the Stadie-Ross procedure, except that 15 cc. samples were analyzed.

indicator. Chloride expressed as milliequivalents per liter is calculated from the equation

$$\text{Chloride} = \frac{1000}{0.5} (0.15 - 0.02 \times \text{cc. KCNS})$$

### Results

The results of a number of analyses of serum are given in Table I, and are compared with analyses according to standard methods. The Stadie-Ross (6) procedure was employed for base, and chlo-

rides were determined by means of Wilson and Ball's modification of Van Slyke's wet ashing method (8, 11). As Table I indicates, the chloride determinations are in very good agreement with the results of the ashing method. The results for total base appear to be approximately 1 per cent higher on the average than those obtained by the Stadie-Ross procedure. Similar results have been obtained by Consolazio (personal communication), who has compared total base determined by the Adair-Keys method with that determined by Hald's benzidine sulfate method (4). He finds the results of the electro-dialytic method to be in good agreement with the gasometric method of Van Slyke, Hiller, and Berthelsen (9), both methods yielding somewhat higher values for total base than those obtained by the analysis of benzidine sulfate. The evidence thus points to a real but not serious discrepancy between the results of the two determinations. The source of this small error is as yet undetermined.

#### SUMMARY

A modification of the Adair-Keys electro-dialytic method for total base is described by which both total base and chloride of serum are simultaneously determined on the same sample.

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